PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : **C12Q 1/68, C12N 5/06, 5/08, 1/16, C07H 21/00**	**A1**	**(11) International Publication Number:** **WO 99/20797** **(43) International Publication Date:** 29 April 1999 (29.04.99)

(21) International Application Number: PCT/US98/22365

(22) International Filing Date: 21 October 1998 (21.10.98)

(30) Priority Data:
08/955,472 21 October 1997 (21.10.97) US

(71) Applicant: UNIVERSITY OF MASSACHUSETTS [US/US]; 26th floor, 1 Beacon Street, Boston, MA 02108 (US).

(72) Inventors: HE, Feng; Apartment #6, 145 Orient Street, Worcester, MA 01604 (US). JACOBSON, Allan, S.; 2 Chestnut Street, Grafton, MA 01519 (US).

(74) Agent: FASSE, J., Peter; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: HETEROLOGOUS POLYPEPTIDE PRODUCTION IN THE ABSENCE OF NONSENSE–MEDIATED mRNA DECAY FUNCTION

(57) Abstract

The invention relates to the discovery of a gene, *NMD2*, named after its role in the Nonsense–Mediated mRNA Decay pathway, and the protein, Nmd2p, encoded by the *NMD2* gene. The amino acid sequence of Nmd2p and the nucleotide sequence of the *NMD2* gene encoding it are disclosed. Nmd2p is shown herein to bind to another protein in the decay pathway, Upf1p. A C–terminal fragment of the protein is also shown to bind Upf1p and, when overexpressed in the host cell, the fragment inhibits the function of Upf1p, thereby inhibiting the nonsense–mediated mRNA decay pathway. The invention also relates to methods of inhibiting the nonsense–mediated mRNA decay pathway to stabilize mRNA transcripts containing a nonsense codon which normally would cause an increase in the transcript decay rate. Such stabilization of a transcript is useful for the production of a recombinant protein or fragment thereof. The invention also relates to methods of identifying molecules that inhibit the nonsense–mediated mRNA decay pathway, and the use of such molecules for treatment of disorders associated with nonsense mutations.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

HETEROLOGOUS POLYPEPTIDE PRODUCTION IN THE ABSENCE OF NONSENSE-MEDIATED mRNA DECAY FUNCTION

Background of the Invention

The invention relates to nonsense-mediated mRNA decay function.

It is well known in the field of biology that changes in the amino acid sequence of a protein can result in changes in the biological function of the protein. To optimize a target biological function, the amino acid sequence can be altered and tested for improved function. In very simple terms, this is the process of evolution by which the proteins that exist naturally today have been selected over eons. It is an advantage of modern molecular biology that such alterations can be made in a matter of days rather than a matter of centuries. Specifically, optimizing the biological function of a protein of pharmaceutical or other commercial interest can be performed by substituting one amino acid for the naturally occurring amino acid at a given site and producing a sufficient quantity of the protein for screening of biological activity.

Production of a recombinant protein in a cellular system requires the efficient translation of the mRNA transcript encoding the protein. For this to occur, the transcript must exist in the cell long enough for translation into the desired recombinant protein. mRNA transcripts vary in the length of time (transcript half-life) that they exist in a cell prior to being degraded by cellular proteins specific for that purpose. In some cases, degradation occurs rapidly such that very little protein is produced.

For example, the yeast cell, *Saccharomyces cerevisiae*, a commonly used cellular system for the

production of recombinant proteins, has a biological pathway that specifically degrades mRNA transcripts containing a non-coding triplet sequence (nonsense or stop codons) in the transcript. In several genes studied thus far, the destabilizing nonsense codon occurs within the 5'-proximal portion of the transcript (reviewed in Peltz et al., Prog. Nucl. Acids Res. Mol. Biol. (1994) 47:271-297). The translation process stops at the nonsense codons prior to reaching the end of the transcript's coding sequence resulting in the production of a truncated protein that may not possess normal biological activity. Thus, the cell has developed a biochemical system to degrade transcripts containing mutations that create stop codons early in the coding sequence.

However, in a cell of a suppressor strain that suppresses nonsense codons, a nonsense codon can be a useful means of coding for an alternate amino acid when a nonsense codon is engineered into the coding sequence to produce an altered protein which is then screened for enhanced biological activity. Suppressor strains (e.g., *SUF1-1*) do not allow maximal expression of a nonsense codon-containing transcript (Leeds et al., (1991) Genes & Dev. 5:2303-2314).

Nonsense-mediated mRNA decay is a phenomenon in which nonsense mutations, e.g., point or frame shift mutations that create a stop codon in the reading frame, in a gene can enhance the decay rate of the mRNA transcribed from that gene. For a review, see, e.g., Peltz et al., (1994) Prog. Nuc. Acid Res. Mol. Biol. 47:271-297. The process occurs in viruses, prokaryotes, and eukaryotes (Leeds (1991), supra; Barker, G.F. and Beemon, K. (1991) Mol. Cell. Biol. 11:2760-2768; Lim, S.-K. and Maquat, L.E. (1992) EMBO J. 11:3271-3278).

In most genetic systems, 61 of the 64 possible codon triplets encode amino acids. The triplets UAA, UAG, and UGA are non-coding (nonsense codons) and promote translational termination (Osawa et al., (1992) Microbiol. Rev. 56:229-264). The polypeptide chain terminating effects of UAA, UAG, and UGA triplets have been amply documented and characterized (Craigen et al., (1990) Mol. Microbiol. 4:861-865).

Nonsense-mediated mRNA decay has been studied extensively in the yeast *Saccharomyces cerevisiae* where it has been shown that degradation of mRNA via this pathway is most likely to occur in the cytoplasm and is linked to translation. Evidence in support of these conclusions includes the following: 1) unstable, nonsense-containing mRNAs are stabilized in a strain harboring an amber suppressor tRNA (Losson and Lacroute, (1979) Proc. Nat'l. Acad. Sci. USA 76:5134-5137; Gozalbo and Hohmann, (1990) Curr. Genet. 17:77-79); 2) nonsense-containing mRNAs are ribosome-associated (Leeds et al., (1991) Genes & Dev. 5:2303-2314; He et al., (1993) Proc. Nat'l. Acad. Sci. USA 90:7034-7039) and the number of ribosomes associated with such mRNAs is a function of the relative positions of the respective nonsense codons (He et al., (1993) Proc. Nat'l. Acad. Sci. USA 90:7034-7039); and 3) treatment of cells with cycloheximide, an inhibitor of translational elongation, stabilizes nonsense-containing mRNAs, yet removal of cycloheximide leads to the immediate restoration of rapid mRNA decay (Peltz et al., (1997) RNA 3:234-244).

Previous studies of nonsense-mediated mRNA decay in yeast also have shown that the products of the *UPF1* and *UPF3* genes (proteins Upf1p and Upf3p, respectively) are essential components of this degradative pathway. Mutations in these genes stabilize mRNAs containing premature nonsense codons without affecting the decay

rates of most wild-type transcripts (Leeds et al., (1991) Genes & Dev. 5:2303-2314, Leeds et al., (1992) Mol. Cell. Biol. 12:2165-2177; Peltz et al., (1993) Genes & Dev. 7:1737-1754; He et al., (1993) Proc. Nat'l. Acad. Sci. USA 90:7034:7039; Cui et al., (1995) Genes & Dev. 9:423-436; He and Jacobson, (1995) Genes & Dev. 9:437-454; He et al., (1997) Mol. Cell. Biol. 17:1580-1594; Lee and Culbertson, (1995) Proc. Nat'l. Acad. Sci. USA 92:10354-10358; Lee and Varmus, (1995) Proc. Nat'l. Acad. Sci. USA 92:6587-6591).

The *UPF1* gene has been cloned and sequenced, (Leeds et al., (1992) Mol. Cell Biol. 12:2165-2177) and shown to be: 1) non-essential for viability; 2) capable of encoding a 109 kD protein with a so-called zinc finger, nucleotide (GTP) binding site, and RNA helicase motifs (Leeds et al., (1992) Mol. Cell. Biol. 12:2165-2177; Altamura et al., (1992) J. Mol. Biol. 224:575-587; Koonin, (1992) Trends Biochem. Sci. 17:495-497); 3) identical to *NAM7*, a nuclear gene that was isolated as a high copy suppressor of mitochondrial RNA splicing mutations (Altamura et al., (1992) J. Mol. Biol. 224:575-587); and 4) partially homologous to the yeast *SEN1* gene (Leeds et al., (1992) Mol. Cell. Biol. 12:2165-2177). The latter encodes a noncatalytic subunit of the tRNA splicing endonuclease complex (Winey and Culbertson, (1988) Genetics 118:607-617; DeMarin et al., (1992) Mol. Cell. Biol. 12:2154-2164), suggesting that the Upf1p protein (Upf1p) may also be part of a nuclease complex targeted specifically to nonsense-containing mRNAs.

Suppression of nonsense-mediated mRNA decay in *upf1* deletion strains does not appear to result simply from enhanced read-through of the termination signal (Leeds et al., (1991) Genes & Dev. 5:2303-2314), nor does it appear to be specific for a single nonsense codon. The ability of *upf1*$^-$ mutants to suppress *tyr*7-1 (UAG),

leu2-1 (UAA), *leu2*-2 (UGA), *met8*-1 (UAG), and *his4*-166 (UGA) (Leeds et al., (1992) Mol. Cell. Biol. 12:2165-2177) indicates that they can act as omnipotent suppressors. *upf1*⁻ mutants degrade nonsense-containing transcripts at a slower rate allowing synthesis of sufficient read-through protein to permit cells to grow under nutrient-deficient conditions that are nonpermissive for *UPF1*⁺ cells.

Summary of the Invention

The invention relates to the discovery of a gene, *NMD2*, named after its role in the Nonsense-Mediated mRNA Decay pathway, and the protein, Nmd2p, encoded by the *NMD2* gene. Nmd2p is shown herein to bind to Upf1p. A C-terminal fragment of the protein is also shown to bind to Upf1p and, when overexpressed in the host cell, the fragment inhibits the function of Upf1p, thereby inhibiting the nonsense-mediated mRNA decay pathway. The components of the nonsense-mediated mRNA decay pathway monitor the fidelity of translation, terminating translation and accelerating decay when a premature nonsense codon-containing mRNA is detected. Interference with the components thus alters both the decay process and the fidelity process. Inhibition of the nonsense-mediated mRNA decay pathway is a useful means of treating disorders caused by the presence of nonsense mutations.

The invention further relates to the inhibition of the nonsense-mediated mRNA pathway to produce a heterologous recombinant protein or polypeptide in a host cell or to increase the production of an endogenous protein useful to a host cell or organism. A codon of the gene encoding the recombinant protein is mutated to encode a nonsense codon. Expression of this recombinant protein is enhanced by stabilizing the nonsense codon-

containing mRNA transcript in a host cell in which the nonsense-mediated mRNA decay pathway is inhibited.

The insertion of a nonsense codon into the gene of interest is useful to produce an altered heterologous protein by amino acid substitution at the nonsense codon in a suppressor host strain. Insertion of a nonsense codon further allows the controlled expression of a protein that may be toxic to the cell by controlling the timing of nonsense-mediated mRNA decay pathway inhibition. Insertion of a nonsense codon also allows the production of an N-terminal fragment of a heterologous protein in increased yield when the nonsense codon-containing transcript is expressed in a host strain that is not a suppressor of nonsense codons.

The invention further provides methods of increasing expression of nonsense codon-containing transcripts by inhibiting the nonsense-mediated mRNA decay pathway by overexpressing the C-terminal fragment of Nmd2p in the same cell that is also expressing the heterologous protein. Overexpression of the C-terminus of Nmd2p is not deleterious to the cell since its expression provides specific stabilization of transcripts having a stop codon early in the transcript and does not affect the stability of other transcripts.

The invention features a method of substantially inhibiting the nonsense-mediated mRNA decay pathway by providing a cell (such as a yeast cell) and mutating the *NMD2* gene such that essentially no functional Nmd2p is produced. For example, an insertional mutation which prevents synthesis of the Nmd2p results in an inhibited nonsense-mediated mRNA decay pathway without affecting the viability of the cell as described herein.

The invention also features a method of substantially inhibiting the nonsense-mediated mRNA decay pathway by providing a cell (such as a yeast cell) and

mutating the *UPF1* gene such that essentially no functional Upf1p is produced. For example, an insertional mutation which prevents synthesis of the Upf1p results in an inhibited nonsense-mediated mRNA decay pathway without affecting the viability of the cell as described herein.

In addition, the invention features a method of inhibiting the nonsense-mediated mRNA decay pathway by providing a cell and transforming the cell with a vector encoding *NMD2* operably linked to regulatory sequences for constitutive or inducible expression of the antisense transcript. Such an antisense transcript hybridizes to essentially all of the *NMD2* sense transcript preventing translation and the production of functional Nmd2p, thereby inhibiting the nonsense-mediated mRNA decay pathway. By "hybridizing to essentially all of the sense *NMD2* transcript" is meant that a sufficient amount of the sense transcript is bound by antisense transcript to inhibit translation such that substantially no functional Nmd2p protein is produced.

The invention features a method of inhibiting the nonsense-mediated mRNA decay pathway by providing a cell and transforming the cell with a vector encoding *UPF1* operably linked to regulatory sequences for constitutive or inducible expression of the antisense transcript. Such antisense transcript hybridizes to a sufficient portion of the *UPF1* sense transcript to prevent translation production of functional Upf1p, thereby inhibiting the nonsense mediated mRNA decay pathway.

The invention also features a substantially pure DNA of the *NMD2* gene, and degenerate variants thereof, involved in the nonsense-mediated mRNA pathway of a cell. The DNA of the invention is at least 90% identical to SEQ ID NO:1, and is preferably from the yeast *Saccharomyces cerevisiae*. The DNA encodes an amino acid sequence of

Nmd2p (SEQ ID NO:2). The amino acid sequence of the invention is at least 90% identical to the amino acid sequence of SEQ ID NO:2.

The invention also features the substantially pure DNA sequence of the 3' terminus (SEQ ID NO:3) of *NMD2*. The 3' terminus encodes the carboxy terminal fragment (SEQ ID NO:4) of Nmd2p, which fragment, when overexpressed in a yeast cell, binds to Upf1p and inhibits the nonsense-mediated mRNA decay pathway.

In addition, the invention features a vector containing a DNA sequence (SEQ ID NO:1) encoding a polypeptide (SEQ ID NO:2). Preferably the coding sequence is under the transcriptional control of regulatory sequences that are activated and deactivated by an externally applied condition such as temperature, or an externally supplied chemical agent. Such control expression systems are well known to those of ordinary skill in the art. Thus, the expression of the DNA is turned on and off as necessary for the controlled (i.e., conditional) inhibition of the nonsense-mediated mRNA pathway.

The invention further features a vector containing a DNA sequence (SEQ ID NO:3) encoding a polypeptide (SEQ ID NO:4) which polypeptide, when overexpressed in a cell, inhibits the nonsense-mediated mRNA decay pathway. Preferably the coding sequence is under the transcriptional control of regulatory sequences that are activated and deactivated by an externally applied condition such as temperature or an externally supplied chemical agent. Thus, the expression of the DNA is turned on and off as necessary for the controlled (i.e., conditional) inhibition of the nonsense-mediated mRNA decay pathway.

The invention also features a host cell containing the DNA of SEQ ID NO:1 or SEQ ID NO:3 or fragments

thereof. The invention also features cells harboring vectors containing the DNA of SEQ ID NO:1 or SEQ ID NO:3 or fragments thereof.

In another embodiment, the invention features substantially pure nonsense-mediated mRNA decay pathway protein, Nmd2p (SEQ ID NO:2), and fragments thereof from a yeast cell, preferably from the genus *Saccharomyces*.

The invention also features a substantially pure nonsense-mediated mRNA decay pathway protein Nmd2p C-terminal fragment (SEQ ID NO:4) and fragments thereof which bind to the nonsense-mediated mRNA decay pathway protein, Upf1p, and which when overexpressed in a cell, substantially inhibit the nonsense-mediated mRNA decay pathway in the cell.

The invention further features a cell containing a vector expressing a polypeptide containing the Nmd2p carboxy terminal fragment (SEQ ID NO:4), which fragment binds to the nonsense-mediated mRNA decay pathway protein, Upf1p, and, when overexpressed in the cell, substantially inhibits the nonsense-mediated mRNA decay pathway in the cell.

In addition, the invention features methods of producing a heterologous polypeptide from an mRNA transcript in which the transcript contains at least one nonsense codon within a transcript destabilizing 5' portion. The method involves providing a cell in which the nonsense-mediated mRNA decay pathway is substantially inhibited by

1) overexpression of a polypeptide containing the Nmd2p carboxy terminal fragment (SEQ ID NO:4); or 2) mutation of *NMD2* or *UPF1* (e.g., insertional mutagenesis) resulting in inhibition of the nonsense-mediated mRNA decay pathway of the cell; or 3) expression of *NMD2* or *UPF1* antisense mRNA which hybridizes to the sense transcript of *NMD2* or *UPF1*, respectively, inhibiting translation and, thereby

inhibiting the nonsense-mediated mRNA decay pathway. Expression in this cell of a nonsense codon-containing gene encoding the heterologous polypeptide provides a transcript whose stability is enhanced at least two-fold compared to a wild-type cell. Translation of the transcript produces the heterologous polypeptide.

In another embodiment, the invention features antibodies that are raised against and bind specifically to Nmd2p, a protein having the amino acid sequence of SEQ ID NO:2, or a polypeptide having the amino acid sequence of SEQ ID NO:4. The antibodies can be polyclonal or monoclonal.

The invention further features a method of screening a candidate host cell for the presence or absence of 1) Nmd2p, 2) a C-terminal fragment of Nmd2p, 3) a polypeptide of SEQ ID NO:2, or 4) a polypeptide of SEQ ID NO:4, including fragments or analogs thereof. The method also can be used to determine relative amounts of each of the proteins in a cell. The screening method is useful for isolating a host strain in which heterologous protein production is to be optimized. The method first involves lysis of a clonal population of cells suspected of containing Nmd2p or Nmd2p fragment. Antibody to Nmd2p or Nmd2p fragment is contacted with proteins of the lysate. Presence, relative abundance, or absence of Nmd2p or Nmd2p fragment in the lysate is determined by the binding of the antibody. Possible detection methods include affinity chromatography, Western blotting, or other techniques well known to those of ordinary skill in the art.

A heterologous polypeptide produced by the methods of the invention can be a particular fragment of a protein or polypeptide. A nonsense codon is incorporated into the DNA sequence encoding the protein or polypeptide at a position within a transcript destabilizing 5'

portion of the sequence at a desired transcriptional stop site. Expression of the DNA in a cell having an inhibited nonsense-mediated mRNA decay pathway results in a substantially increased half-life for the nonsense codon-containing transcript. An advantage of this method is the stabilization of the transcript allowing an increased amount of the protein fragment to be produced relative to the amount produced in a wild-type host strain.

A heterologous protein that is normally toxic to a cell is produced by controllably inhibiting the nonsense-mediated mRNA decay pathway and thereby, controlling the stability of a nonsense codon-containing transcript for the toxic protein. Inhibition of the nonsense-mediated mRNA decay pathway is accomplished, for example, by the inducible expression of the C-terminus of the Nmd2p only when protein production is desired (e.g., at optimal cell density of the culture). Inhibition of the nonsense-mediated mRNA decay pathway substantially increases the half-life of the transcript containing a nonsense codon in a transcript destabilizing 5' portion of the transcript thereby increasing translation and production of the protein when desired. The cell expressing the heterologous protein can be a nonsense suppressor cell in which the suppressor mechanism is controllably expressed and substitutes the naturally occurring amino acid at the site of a nonsense codon.

An altered heterologous polypeptide is produced in a nonsense suppressor cell by substituting an amino acid at the position of a nonsense codon, which amino acid does not naturally occur at that position. An amino acid is substituted which alters a target biological activity of the protein in the cell. The nonsense-mediated mRNA pathway is inhibited to increase production of the altered heterologous polypeptide from a transcript

containing a nonsense codon in a transcript destabilizing 5′ portion of the transcript.

Alteration in biological activity includes increased binding affinity to a target molecule such as a receptor, antibody, or decreased toxicity of the protein to the host strain in which the protein is produced. By "substantial reduction in toxicity" is meant that expression of the altered heterologous polypeptide allows the cell growth rate to be at least two-fold greater than the growth rate in the presence of the natural toxic heterologous polypeptide, or allows sufficient cell growth for production of the altered heterologous protein.

An advantage of the invention is the ability to increase heterologous protein production and direct amino acid substitution to a desired codon position using a nonsense codon and producing the protein in a suppressor mutant such that a known amino acid is substituted in each suppressor host. Stabilization of the mRNA transcript by inhibiting the nonsense-mediated mRNA decay pathway increases the half-life of the transcript (decreases its decay rate) thereby allowing increased translation from the transcript. Preferably the nonsense codon is present in a transcript destabilizing 5′ portion of the transcript. Preferably the transcript containing the nonsense codon decays rapidly in the presence of an unaltered wild-type nonsense-mediated mRNA decay pathway, and decays at least two-fold more slowly in the presence of a nonsense-mediated mRNA decay pathway inhibited by the method of the invention.

The invention also includes a substantially pure polypeptide that specifically binds to the Upf1p protein, wherein the binding causes inhibition of the nonsense mediated mRNA decay pathway.

In another embodiment, the invention features substantially pure nucleic acids (and vectors containing them) which hybridize under stringent conditions to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3 or their complementary sequences, wherein the nucleic acid encode an Nmd2p polypeptide or a carboxy terminal fragment of an Nmd2p polypeptide that inhibits the nonsense-mediated mRNA decay pathway in a cell, respectively.

In yet another aspect, the invention features a method of determining whether a candidate compound, e.g., a small molecule or nucleic acid, modulates the nonsense-mediated mRNA decay pathway by a) obtaining a cell (e.g., from a mammal such as a human) containing a mutation in a specific nonsense mutation-containing gene; b) incubating the cell with the candidate compound under conditions and for a time sufficient for the cell to express nonsense-mediated mRNA decay pathway genes in the absence of the candidate compound; and c) measuring expression (e.g., RNA or protein) of the nonsense mutation-containing gene, or activity of the gene product in the presence and in the absence of the candidate compound, wherein a difference in expression or activity indicates that the compound modulates nonsense-mediated mRNA decay. The cell can be, for example, a yeast cell containing a nonsense mutation in a gene such that the ability of the cell to grow in a selective medium depends on the functionality of the nonsense-mediated decay pathway. Further, the gene containing the nonsense mutation can be selected from the group consisting of tyr7, leu2, and CAN1, and the the nonsense-mediated decay pathway gene can be NMD2, UPF1, UPF3, RENT1, HUPF1, or homologs thereof.

In another aspect, the invention features a method for treating a mammal, e.g., a human, having a disorder

involving a nonsense mutation by administering to the mammal a therapeutically effective amount of a compound that inhibits the nonsense-mediated mRNA decay pathway. For example, the compound can cause decreased expression of UPF1, UPF3, NMD2, RENT1, HUPF1, or their homologs, or decreased activity of Upf1p, Upf3p, or Nmd2p or their homologs. The compound can be the C-terminal fragment of Nmd2p or an antisense oligonucleotide. The disorder can be breast cancer, polycystic kidney disease I, polycystic kidney disease II, Niemann-Pick disease, adenomatous polyposis coli, cystic fibrosis, Fanconi's anemia, hemophilia, hypercholesterolemia, neurofibromatosis, ornithine transcarbamylase deficiency, retinoblastoma, glycogen storage disease, McArdle disease, cancer, Tay-Sachs disease, Cowden disease, Wilson disease, or β-thalassaemia.

The invention also features method for treating a patient with a disorder associated with excessive expression or activity of an NMD2 gene, the method involving administering to the patient a compound which inhibits expression of NMD2.

A "substantially pure DNA" is a DNA that is not immediately contiguous with (i.e., covalently linked to) both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR (polymerase chain reaction) or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is

part of a hybrid gene encoding additional polypeptide sequences.

A "polypeptide" is any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation).

By "inhibited nonsense-mediated mRNA decay pathway" is meant decreased turnover of a nonsense codon-containing mRNA transcript in which the half-life of the nonsense codon-containing mRNA is at least two-fold greater in a nonsense-mediated mRNA decay pathway altered by the methods of the invention relative to its half-life in a wild type cell. Techniques for measuring mRNA half-life are described herein and in Parker et al. (1991) Meth. Enzymol. 194:415-423. The pathway can also be inhibited by increased read-through of nonsense codon-containing mRNAs.

A "transcript destabilizing 5′ portion" is a 5′ proximal region of an mRNA transcript in which region the presence of a nonsense codon results in an increased rate of transcript degradation by at least two-fold compared to the normal transcript in a wild-type organism. Determination of a transcript destabilizing 5′ portion is readily performed by one of ordinary skill in the art. The half-life of the transcript from each altered DNA is compared to the wild-type transcript by standard techniques. An approximately two-fold or more decrease in half-life for the altered transcript in a cell expressing wild-type nonsense-mediated mRNA decay pathway activity indicates that the nonsense codon is in a transcript destabilizing region. The region 5′ proximal of the most downstream destabilizing nonsense codon position is considered a transcript destabilizing 5′ portion.

"Nmd2p" is the protein encoded by a gene, NMD2, which is involved in the nonsense-mediated mRNA decay

pathway (e.g., SEQ ID NO:1 depicts the NMD2 gene of *Saccharomyces cerevisiae* which encodes the Nmd2p depicted in SEQ ID NO:2).

"Upf1p" is the protein encoded by a gene, UPF1, which is involved in the nonsense-mediated mRNA decay pathway (e.g., Figs. 5A and 5B (SEQ ID NO:7) depicts a *UPF1* nucleic acid sequence of *Saccharomyces cerevisiae* which encodes the Upf1p depicted in Fig. 6 (SEQ ID NO:8); GenBank Accession No. M76659; Leeds et al. (1992), supra).

"Upf3p" is the protein encoded by a gene, UPF3, which is involved in the nonsense-mediated mRNA decay pathway (e.g., Fig. 7 (SEQ ID NO:9) depicts a *UPF3* nucleic acid sequence of *Saccharomyces cerevisiae* which encodes the Upf3p depicted in Fig. 8 (SEQ ID NO:10); GenBank Accession No. L41153; Lee and Culbertson (1995), supra).

A "substantially pure polypeptide" is a polypeptide, e.g., a nonsense-mediated mRNA decay pathway polypeptide or fragment thereof, that is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, nonsense-mediated mRNA decay pathway polypeptide or fragment. A substantially pure nonsense-mediated mRNA decay pathway polypeptide or fragment thereof is obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid encoding a nonsense-mediated mRNA decay pathway polypeptide or fragment thereof; or by chemically synthesizing the polypeptide or fragment. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A "carboxy terminal fragment of Nmd2p" is the sequence including amino acid 326 to amino acid 1089 (SEQ ID NO:4) or a fragment thereof. The carboxyl terminus is any polypeptide including SEQ ID NO:4 or a fragment thereof that substantially inhibits nonsense-mediated mRNA decay in a cell when the fragment is expressed above endogenous level, as described herein.

By "substantially inhibit nonsense-mediated mRNA decay" is meant to cause an increase by at least two-fold in the half-life of an mRNA of interest in the presence of an inhibiting agent (e.g., a chemical agent, a polypeptide fragment, or like substance) that interferes with the functioning of the proteins of the nonsense-mediated mRNA pathway.

An "overexpressed polypeptide" is a polypeptide which, when produced by the *in vivo* expression of a DNA sequence to produce that polypeptide, is produced in a quantity at least two-fold greater than the quantity of the same polypeptide expressed from the endogenous transcription /translation regulatory elements of the DNA sequence of interest. In the case of the expression of a gene fragment, the endogenous regulatory elements are those of the native gene.

By "substantially increased transcript stability" is meant an increase in the half-life of an mRNA transcript by at least two-fold in the presence of an inhibited nonsense-mediated mRNA decay pathway. The half-life of an mRNA transcript can be measured by extracting at various time points total mRNA from a cell expressing the gene of interest. This is followed by determining the abundance of a transcript over time by Northern analysis using a labelled (e.g., radiolabelled probe) nucleic acid probe to visualize the transcript. Increased transcript stability can also be inferred from increased expression of a polypeptide from the gene of

interest in the presence of an inhibited nonsense-mediated mRNA pathway.

By "essentially no functional protein produced" is meant that a particular protein (e.g., Nmd2p or Upf1p) is present in a cell in such low amounts that the nonsense-mediated mRNA decay pathway is inhibited, resulting in at least a two-fold increase in the stability of mRNA transcripts containing a nonsense codon in a transcript destabilizing 5' portion.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

"Inducible regulatory sequences" are regulatory sequences (e.g., transcriptional regulatory sequences) whose function is initiated by the introduction of one or more external agents to the cell culture medium and whose function is inhibited by the removal of the external agents.

By "specifically binds" is meant a molecule that binds to a particular entity, e.g., an Nmd2p polypeptide, but which does not substantially recognize or bind to other molecules in a sample, e.g., a biological sample, which includes the particular entity, e.g., Nmd2p.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and

examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Description of the Drawings

Figs. 1A to 1C are a representation of the DNA sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of NMD2. Cloning of the *NMD2* gene and determination of its DNA sequence are described herein. The predicted amino acid sequence is indicated in single-letter code and shown below each line of DNA sequence. Position number 1 corresponds to the A of the ATG initiation codon. The *NMD2* open reading frame is interrupted by an intron of 113 nucleotides in which the conserved 5′ splice site [GUAUGU], branchpoint [UACUAAC], and 3′ splice site [AG] are underlined. Transcription initiation sites at nucleotides -56, -60, -64, and -67 (relative to the initiator ATG) were determined by primer extension analysis and are indicated by vertical arrows. The putative TATA box and Abf1p binding consensus sequence, located between positions -219 to -213 and -198 to -186 in the *NMD2* promoter region are respectively underlined by dashed lines. Double underlined residues fit the consensus for a bipartite nuclear localization signal (Dingwall and Laskey, (1991) Trends Biochem. Sci. 16:478-481). The positions where FLAG- or MYC-epitope tag sequences were inserted are indicated by lollipops and the position where the original *GAL4-NMD2* fusion begins is indicated by an arrow with a right angle stem. The bent arrow also indicates the start of the DNA sequence from nucleotide 1089 to nucleotide 3383 (SEQ ID NO:3) encoding the carboxyl terminal amino acid sequence from amino acid 326 to amino

acid 1089 (SEQ ID NO:4) of Nmd2p, a peptide fragment which, when overexpressed, binds to Upf1p and inhibits the nonsense-mediated mRNA decay pathway.

Figs. 2A to 2C are diagrams illustrating insertion and deletion experiments performed to assess the active regions of *NMD2* gene. DNA fragments associated with *NMD2* function are indicated. Fig. 2A is a restriction map of the *nmd2::HIS3* allele. Fig. 2B is a restriction map of the *NMD2* gene. Fig. 2C is a diagram of the results of a complementation analysis to determine functional portions of Nmd2p.

Figs. 3A to 3C are representations of autoradiograms. Fig. 3A is reproduced from a Southern analysis of wild type and HIS3-disrupted NMD2 associated with *NMD2* gene disruption. Fig. 3B is reproduced from a Northern analysis of the stability of different nonsense-containing *PGK1* alleles in *NMD2* and *nmd2::HIS3* haploid yeast strains. Fig. 3C is reproduced from a Northern analysis of *CYH2* pre-mRNA and mRNA transcript stability.

Figs. 4A to 4B are representations of Northern analysis autoradiograms which record the *CYH2* transcript stability phenotypes associated with disruption of both the *NMD2* and *UPF1* genes or overexpression of Nmd2p fragments.

Figs. 5A and 5B are a representation of the nucleic acid sequence of UPF1 (SEQ ID NO:7).

Fig. 6 is a representation of the deduced amino acid sequence of Upf1p (SEQ ID NO:8).

Fig. 7 is a representation of the nucleic acid sequence of UPF3 (SEQ ID NO:9).

Fig. 8 is a representation of the deduced amino acid sequence of Upf3p (SEQ ID NO:10).

Detailed Description

This invention relates to a DNA sequence, a protein, and methods useful in inhibiting the nonsense-mediated mRNA decay pathway in a cell, preferably in a yeast cell or a human cell, e.g., by stabilizing an mRNA transcript which contains a nonsense codon. Preferably, the nonsense codon is in a transcript destabilizing 5' portion of the transcript. Stabilization of the transcript allows increased translation and increased production of a heterologous protein of interest. The protein of interest can be a full-length protein if the nonsense codon is suppressed. The protein of interest can be a desired N-terminal fragment of a protein if the nonsense codon is not suppressed.

Inhibition of the decay of transcripts from the nonsense mutation-containing gene can ameliorate the effects of disorders caused by the presence of a nonsense codon. This can be accomplished by inhibiting a component of the nonsense-mediated decay pathway (e.g., Nmd2p, Upf1p, or Upf3p) with, for example, compounds that bind to Nmd2p, compounds that interfere with the interaction between NMD2 and other molecules in the nonsense-mediated RNA decay pathway (e.g., Upf1p or Upf3p), or compounds that inhibit the expression of nonsense-mediated mRNA decay pathway genes. Antisense therapy or ribozyme therapy are other methods of inhibiting the expression of components of the nonsense mediated decay pathway.

Antisense Constructs and Therapies

Treatment regimes based on an "antisense" approach involve the design of oligonucleotides (either DNA or RNA) that are complementary to nonsense-mediated mRNA decay pathway mRNAs (e.g., transcripts from NMD2 or UPF1). These oligonucleotides bind to the complementary

mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, is a sequence sufficiently complementary to be able to hybridize with the RNA, forming a stable duplex, within the environment of a cell; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The invention also encompasses nucleic acid molecules (DNA and RNA) that hybridize under stringent conditions to a nucleic acid molecule encoding a nonsense-mediated decay pathway polypeptide. The cDNA sequences described herein can be used to identify these nucleic acids, which include, for example, nucleic acids that encode homologous polypeptides in other species, and splice variants of nonsense-mediated decay pathway genes (e.g., an NMD2) in humans or other mammals. Accordingly, the invention features methods of detecting and isolating these nucleic acid molecules. Using these methods, a sample (for example, a nucleic acid library, such as a cDNA or genomic library) is contacted (or "screened") with an NMD2-specific probe (for example, a fragment of SEQ ID NO:1 that is at least 25 or 50 nucleotides long). The probe will selectively hybridize to nucleic acids encoding related polypeptides (or to complementary

sequences thereof). The term "selectively hybridize" is used to refer to an event in which a probe binds to nucleic acids encoding a nonsense-mediated mRNA decay pathway gene such as NMD2 (or to complementary sequences thereof) to a detectably greater extent than to nucleic acids encoding other proteins (or to complementary sequences thereof). The probe, which can contain at least 25 (for example, 25, 50, 100, or 200 nucleotides) can be produced using any of several standard methods (see, for example, Ausubel et al.,"Current Protocols in Molecular Biology, Vol. I," Green Publishing Associates, Inc., and John Wiley & Sons, Inc., NY, 1989). For example, the probe can be generated using PCR amplification methods in which oligonucleotide primers are used to amplify an NMD2-specific nucleic acid sequence (for example, a nucleic acid encoding the chemokine-like domain) that can be used as a probe to screen a nucleic acid library and thereby detect nucleic acid molecules (within the library) that hybridize to the probe.

One single-stranded nucleic acid is said to hybridize to another if a duplex forms between them. This occurs when one nucleic acid contains a sequence that is the reverse and complement of the other (this same arrangement gives rise to the natural interaction between the sense and antisense strands of DNA in the genome and underlies the configuration of the "double helix"). Complete complementarity between the hybridizing regions is not required in order for a duplex to form; it is only necessary that the number of paired bases is sufficient to maintain the duplex under the hybridization conditions used.

Typically, hybridization conditions are of low to moderate stringency. These conditions favor specific interactions between completely complementary sequences,

but allow some non-specific interaction between less than perfectly matched sequences to occur as well. After hybridization, the nucleic acids can be "washed" under moderate or high conditions of stringency to dissociate duplexes that are bound together by some non-specific interaction (the nucleic acids that form these duplexes are thus not completely complementary).

As is known in the art, the optimal conditions for washing are determined empirically, often by gradually increasing the stringency. The parameters that can be changed to affect stringency include, primarily, temperature and salt concentration. In general, the lower the salt concentration and the higher the temperature, the higher the stringency. Washing can be initiated at a low temperature (for example, room temperature) using a solution containing a salt concentration that is equivalent to or lower than that of the hybridization solution. Subsequent washing can be carried out using progressively warmer solutions having the same salt concentration. As alternatives, the salt concentration can be lowered and the temperature maintained in the washing step, or the salt concentration can be lowered and the temperature increased. Additional parameters can also be altered. For example, use of a destabilizing agent, such as formamide, alters the stringency conditions.

In reactions where nucleic acids are hybridized, the conditions used to achieve a given level of stringency will vary. There is not one set of conditions, for example, that will allow duplexes to form between all nucleic acids that are 85% identical to one another; hybridization also depends on unique features of each nucleic acid. The length of the sequence, the composition of the sequence (for example, the content of purine-like nucleotides versus the content of pyrimidine-

like nucleotides) and the type of nucleic acid (for example, DNA or RNA) affect hybridization. An additional consideration is whether one of the nucleic acids is immobilized (for example, on a filter).

An example of a progression from lower to higher stringency conditions is the following, where the salt content is given as the relative abundance of SSC (a salt solution containing sodium chloride and sodium citrate; 2X SSC is 10-fold more concentrated than 0.2X SSC). Nucleic acids are hybridized at 42°C in 2X SSC/0.1% SDS (sodium dodecylsulfate; a detergent) and then washed in 0.2X SSC/0.1% SDS at room temperature (for conditions of low stringency); 0.2X SSC/0.1% SDS at 42°C (for conditions of moderate stringency); and 0.1X SSC at 68°C (for conditions of high stringency). Washing can be carried out using only one of the conditions given, or each of the conditions can be used (for example, washing for 10-15 minutes each in the order listed above). Any or all of the washes can be repeated. As mentioned above, optimal conditions will vary and can be determined empirically.

A second set of conditions that are considered "stringent conditions" are those in which hybridization is carried out at 50°C in Church buffer (7% SDS, 0.5% $NaHPO_4$, 1 M EDTA, 1% BSA) and washing is carried out at 50°C in 2X SSC.

Where a particular polypeptide or nucleic acid molecule is said to have a specific percent identity to a reference polypeptide or nucleic acid molecule of a defined length, the percent identity is relative to the reference polypeptide or nucleic acid molecule. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might

also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria. The same rule applies for nucleic acid molecules.

For polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids, 50 amino acids, or 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 100 nucleotides or 300 nucleotides.

In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Sequence identity can be measured using sequence analysis software (for example, the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705), with the default parameters as specified therein.

Oligonucleotides that are complementary to the 5′ end of the message, e.g., the 5′ untranslated sequence, up to and including the AUG initiation codon, are generally most efficient for inhibiting translation.

However, sequences complementary to the 3′ untranslated sequences of mRNAs have also been shown to be effective for inhibiting translation (Wagner, *Nature*, 372:333, 1984). Thus, oligonucleotides complementary to either the 5′ or 3′ non-translated, non-coding regions of a nonsense-mediated mRNA decay gene, e.g., the human homolog of NMD2, could be used in an antisense approach to inhibit translation of the endogenous human homolog of NMD2 mRNA. Oligonucleotides complementary to the 5′ untranslated region of the mRNA should include the complement of the AUG start codon.

Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation, but could be used in accordance with the invention. Whether designed to hybridize to the 5′, 3′, or coding region of a nonsense-mediated mRNA decay pathway mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, or at least 50 nucleotides in length.

Regardless of the choice of target sequence, *in vitro* studies are usually performed first to assess the ability of an antisense oligonucleotide to inhibit gene expression. In general, these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. In these studies levels of the target RNA or protein are usually compared with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same

length as the test oligonucleotide, and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule or hybridization. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (as described, e.g., in Letsinger et al., *Proc. Nat'l. Acad. Sci. USA* 86:6553, 1989; Lemaitre et al., *Proc. Nat'l. Acad. Sci. USA* 84:648, 1987; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, for example, PCT Publication No. WO 89/10134), or hybridization-triggered cleavage agents (see, for example, Krol et al., *BioTechniques* 6:958, 1988), or intercalating agents (see, for example, Zon, *Pharm. Res.* 5:539, 1988). To this end, the oligonucleotide can be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethyl-aminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,

2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-theouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 2-(3-amino-3-N-2-carboxypropl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

The antisense oligonucleotide may also include at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal, or an analog of any of these backbones.

The antisense oligonucleotide can include an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., *Nucl. Acids. Res.* 15:6625, 1987). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., *Nucl. Acids Res.* 15:6131, 1987), or a chimeric RNA-DNA analog (Inoue et al., *FEBS Lett.* 215:327, 1987).

Antisense oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are

commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (*Nucl. Acids Res.* 16:3209, 1988), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., *Proc. Nat'l. Acad. Sci. USA* 85:7448, 1988).

While antisense nucleotides complementary to the coding region of a nonsense-mediated mRNA decay gene could be used, those complementary to the transcribed untranslated region are most preferred. These include antisense oligonucleotides, 20-30 nucleotides in length, complementary to sequences downstream of the cap site or 5′ to the initiator AUG of the respective mRNAs. In yeast NMD2 mRNA, these regions include the mRNA sequences AAUGCUUAAAUAAUCUAAUAUUGUAUCUGC (SEQ ID NO:11) and UCUGCAUUGAUAAUAUCAUUGGACAGAAAUU (SEQ ID NO:12; He and Jacobson, Genes & Dev. 9: 437-454, 1995). In the human UPF1 homologs, (RENT1; HUPF1), these regions include the sequences GGCGGCUCGGCACUGUUACCUCUCGGUCCG (SEQ ID NO:13) and AACCGGCCCGAGGGCCCUACCCGGAGGCACC (SEQ ID NO:14) ; Perlick et al., (1996) Proc. Nat. Acad. Sci. USA 93:10928-10932, 1996; Applequist et al., (1997) Nucleic Acids Res. 25:814-821).

The antisense molecules should be delivered to cells that express nonsense-mediated mRNA decay proteins *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

However, it is often difficult to achieve intracellular concentrations of the antisense molecule sufficient to suppress translation of endogenous mRNAs. Therefore, an approach may be used in which a recombinant DNA construct comprises an antisense oligonucleotide placed under the control of a strong *pol* III or *pol* II promoter. The use of such a construct to transfect target cells in a patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous nonsense-mediated mRNA decay pathway transcript and thereby prevent translation of that mRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA.

Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Suitable promoters may include, but are not limited to: the SV40 early promoter region (Bernoist et al., *Nature* 290:304, 1981); the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., *Cell* 22:787-797, 1988); the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci. USA* 78:1441, 1981); or the regulatory sequences of the metallothionein gene (Brinster et al., *Nature* 296:39, 1988). Constructs may also be contained on an artifical chromosome (Huxley, Trends. Genet. 13:345-347, 1997).

The production of an NMD2 antisense nucleic acid molecule by any gene therapeutic approach described above results in a cellular level of Nmd2p that is less than the amount present in an untreated individual.

Ribozymes

Ribozyme molecules designed to catalytically cleave nonsense-mediated mRNA decay pathway mRNAs (e.g., an NMD2 mRNA) can also be used to prevent translation of these mRNAs and expression of nonsense-mediated mRNA decay pathway mRNAs (see, e.g., PCT Publication WO 90/11364; Saraver et al., *Science* 247:1222, 1990). While various ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy specific mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5′-UG-3′. The construction and production of hammerhead ribozymes is well known in the art (Haseloff et al., *Nature* 334:585, 1988). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5′ end of the nonsense-mediated mRNA decay mRNA, i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

Potential ribozyme sites in a nonsense-mediated mRNA decay pathway protein include 5′-UG-3′ sites which correspond to the initiator methionine codon. UG-containing sequences are located throughout the yeast NMD2 mRNA, including those surrounding codon 3 (AGGAUGGACG) (SEQ ID NO:15), codons 17-18 (CUUGGAAUGGCGAAGAA)(SEQ ID NO:16), codon 121

(CUUUUGAGAAC)(SEQ ID NO:17), codon 203 (UAUUGCGA), and codon 404 (AUAUUUGGACAA)(SEQ ID NO:18), among others.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes"), such as the one that occurs naturally in *Tetrahymena Thermophila* (known as the IVS or L-19 IVS RNA), and which has been extensively described by Cech and his collaborators (Zaug et al., *Science* 224:574, 1984; Zaug et al., *Science*, 231:470, 1986; Zug et al., *Nature* 324:429, 1986; PCT Application No. WO 88/04300; and Been et al., *Cell* 47:207, 1986). The Cech-type ribozymes have an eight base-pair sequence that hybridizes to a target RNA sequence, whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences present in nonsense-mediated mRNA decay pathway proteins.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.), and should be delivered to cells which express a nonsense-mediated mRNA decay pathway gene *in vivo*, e.g., heart, skeletal muscle, thymus, spleen, and small intestine. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive *pol* III or *pol* II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous nonsense-mediated mRNA decay pathway messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

For any of the above approaches, the therapeutic NMD2 antisense or ribozyme nucleic acid molecule construct is preferably applied to the site of the target area (for example, a hematopoetic stem cell in the case

of β-thalassemia, delivered by injection), but can also be applied to tissue in the vicinity of the target area or even to a blood vessel supplying the target area.

For gene therapy, antisense or ribozyme NMD2 expression is directed from any suitable promoter (e.g., the human cytomegalovirus, simian virus 40, or metallothionein promoters), and its production is regulated by any desired mammalian regulatory element. For example, if desired, enhancers known to direct preferential gene expression in hematopoetic stem cells can be used to direct antisense NMD2 expression in a patient with β-thalassemia.

NMD2 antisense or ribozyme therapy is also accomplished by direct administration of an antisense NMD2 or ribozyme RNA to a target area. This mRNA can be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense NMD2 DNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense NMD2 RNA to target cells is carried out by any of the methods for direct nucleic acid administration described above.

Other Methods for Reducing Nonsense-mediated mRNA Decay Pathway Expression

Endogenous nonsense-mediated mRNA decay can also be reduced by inactivating or "knocking out" the nonsense-mediated mRNA decay pathway gene or its promoter using targeted homologous recombination (see, e.g., U.S. Patent No. 5,464,764). For example, a mutant, non-functional NMD2 nucleic acid sequence (or a completely unrelated DNA sequence) flanked by DNA homologous to the NMD2 gene (either the coding regions or regulatory regions of the NMD2 gene) can be used, with or without a selectable marker and/or a negative selectable marker, to

transfect cells that express NMD2 *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the NMD2 gene. Such approaches are particularly suited for use in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive nonsense-mediated mRNA decay. However, this approach can be adapted for use in humans. For example, the recombinant DNA constructs may be directly administered or targeted to the pertinant cells *in vivo* using appropriate viral vectors.

Alternatively, endogenous nonsense-mediated mRNA decay pathway gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the nonsense-mediated mRNA decay pathway gene (*i.e.*, an NMD2 promoter and/or enhancers) to form triple helical structures that prevent transcription of an NMD2 gene in target cells in the body (Helene, *Anticancer Drug Res.* 6:569, 1981; Helene et al., *Ann. N.Y. Acad. Sci.* 660:27, 1992; and Maher, *Bioassays* 14:807, 1992).

Vectors

Vectors to be used as described above include retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for Nmd2p-expressing cells (e.g., cells with activated nonsense-mediated mRNA decay pathways) can be used as a gene transfer delivery system for a therapeutic antisense nucleic acid construct or other nucleic acid construct that inhibits expression of a nonsense-mediated mRNA decay pathway gene (e.g., NMD2) expression. Numerous vectors useful for this purpose are generally known [Miller, *Human Gene Therapy* 15-14, (1990); Friedman, *Science* 244:1275-1281, (1989); Eglitis

and Anderson, *BioTechniques* 6:608-614, (1988); Tolstoshev and Anderson, *Current Opinion in Biotechnology* 1:55-61, (1990); Sharp, *The Lancet* 337:1277-1278, (1991); Cornetta et al., *Nucleic Acid Research and Molecular Biology* 36:311-322, (1987); Anderson, *Science* 226:401-409, (1984); Moen, *Blood Cells* 17:407-416, (1991); and Miller and Rosman, *BioTechniques* 7:980-990, (1989); Le Gal La Salle et al., *Science* 259:988-990, (1993); and Johnson, Chest 107:77S-83S, (1995)]. Retroviral vectors are particularly well developed and have been used in clinical settings [Rosenberg et al., *N. Engl. J. Med* 323:370, (1990); Anderson et al., U.S. Pat. No. 5,399,346].

Non-viral approaches can also be employed for the introduction of therapeutic DNA into malignant cells. For example, an antisense NMD2 nucleic acid can be introduced into a carcinoma cell by the techniques of lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413, (1987); Ono et al., *Neurosci. Lett.* 117:259, (1990); Brigham et al., *Am. J. Med. Sci.* 298:278, (1989); Staubinger and Papahadjopoulos, *Meth. Enz.* 101:512, 1983); polylysine conjugation methods (Wu and Wu, *J. Biol. Chem.* 263:14621, 1988; Wu et al., *J. Biol. Chem.* 264:16985, 1989); or, by microinjection under surgical conditions (Wolff et al., *Science* 247:1465, 1990).

Examples

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

The examples illustrate the invention by describing the *NMD2* gene, the Nmd2 protein, and its C-terminal fragment. Methods of substantially inhibiting the nonsense-mediated mRNA decay pathway in a cell, and methods of producing heterologous proteins and fragments

of proteins are also described. These methods can inhibit the nonsense-mediated mRNA decay pathway to increase transcript stability. Other aspects the nonsense-mediated mRNA decay pathway can be affected, e.g., there can be increased read-through of nonsense codon-containing mRNAs. Inhibition of the nonsense-mediated mRNA decay pathway is useful for treating disorders involving a nonsense mutation.

Example 1: Identification of a Gene Encoding a Putative Upf1p-interacting Protein

To identify a gene or genes encoding putative Upf1p-interacting proteins, the yeast two-hybrid system was used. This method of detecting protein-protein interactions in yeast is based on the observation that the DNA binding and transcriptional activation functions of the *GAL4* protein (Gal4p) can reside on two distinct chimeric polypeptides and still activate transcription from a *GAL* UAS (Upstream Activating Sequence), provided that the two polypeptides can interact with each other (Fields and Song, (1989) Nature 340:245-246; Chien, C.-T. et al., (1991) Proc. Natl. Acad. Sci. 88:9578-9582). As employed herein, the first hybrid was cloned into a plasmid (such as pMA424; (Ma, J. and Ptashne, M. (1988) Cell 55:443-446) in which the entire *UPF1* coding region was fused in-frame to the Gal4p DNA binding domain (amino acids 1-147 of Gal4p). Construction of plasmid pMA424-*UPF1* was performed by a three-fragment ligation. A fragment of 144 bp from the initial ATG codon to the 48th codon of *UPF1* was amplified by the polymerase chain reaction (PCR) using UPF1-TH-5′ (SEQ ID NO:5) and UPF1-TH-3′ (SEQ ID NO:6) as oligonucleotide primers (Table 1).

TABLE 1 - Oligonucleotide Primers

UPF-TH-5' 5'-CCGGAATTCATGGTCGGTTCCGGTTCT-3' (SEQ ID NO:5)

UPF-TH-3' 5'-AGTGACTTGAGCCTC-3' (SEQ ID NO:6)

Amplification with these primers led to the introduction of an *Eco*RI site adjacent to the initiator ATG. The PCR-amplified fragment was digested with *Eco*RI and *Bst*XI and ligated with a *Bst*XI-*Bam*HI fragment (including the rest of the *UPF1* coding region and approximately 1 kb 3' distal to the translational termination site including the entire 3'UTR) into plasmid pMA424 digested by *Eco*RI and *Bam*HI. DNA sequence analysis confirmed the primary structure of the construct.

Second hybrids were encoded by *S. cerevisiae* genomic DNA libraries in plasmids pGAD(1-3) (Chien et al. (1991) Proc. Nat'l. Acad. Sci USA 88:9578-9582) fused, in the three reading frames, to sequences encoding the Gal4p transcriptional activation domain (amino acids 768-881). Both were cotransformed into a *Saccharomyces cerevisiae* strain that contained an integrated *GAL1-LacZ* reporter construct (such as the *S. cerevisiae* strain GGY1::171 (*Δgal4 Δgal80 URA3::GAL1-LacZ his3 leu2*)) (Gill and Ptashne (1987) Cell 51:121-126) or equivalent strain well known to those of ordinary skill in the art of yeast genetics.

In performing the two-hybrid screening method, the GGY1::171 yeast strain was cotransformed with both pMA424-*UPF1* and a library containing genomic DNA fragments fused to the *GAL4* activation domain. After 3-4 days of growth on SD-His-Leu plates at 30°C, His^+Leu^+ transformants were replica-plated to SSX plates and were incubated until blue colonies appeared as described in Rose et al. (1990) Methods in Yeast Genetics: A

Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). False positive colonies due to cloning of the GAL4 gene into the pGAD vectors were eliminated by PCR yeast cellular DNA using the *GAL4*-specific primers GAL4-5' (from nucleotide 1206 to 1229 of the *GAL4* gene) and GAL4-3' (from nucleotide 2552 to 2528 of the *GAL4* gene)(Laughon and Gesteland, (1984) Mol. Cell Biol. 4:260-267). Cells from the remaining blue colonies were grown in SD-Leu medium and plasmids were recovered and transformed into the *E. coli* strain MH6 by electroporation. The activation domain (pGAD) plasmids from the library were identified by their ability to complement an *E. coli leuB* mutation due to the presence of the plasmid-borne *LEU2* gene. According to the two-hybrid test, transcriptional activation depends interaction between the *UPF1* fusion product and the test fragment fusion product. To confirm that transcriptional activation was dependent on the presence of both gene fusions, the isolated library plasmids were retransformed into the original GGY1::171 strain with either: 1) pMA424-*UPF1*, a *GAL4* DNA-binding domain-*UPF1* fusion plasmid; 2) pMA424, the *GAL4* DNA binding domain vector only; 3) pMA424-*CEP1*, a *GAL4* DNA-binding domain-*CEP1* fusion plasmid; or 4) pMA424-*LAM5*, a *GAL4* DNA-binding domain-*LAM5* fusion plasmid, where *CEP1* and *LAM5* genes are negative control genes whose gene products are known not to bind to *UPF1* gene product. Plasmids that yielded blue colonies only with the pMA424-*UPF1* fusion were characterized further by restriction mapping, Southern analysis, and sequence analysis (see e.g., Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). DNA sequences were compared to existing sequence databases using the FASTA program (Devereux et al., (1984) Nucleic Acids Res. 12:387-395). Colonies

expressing detectable β-galactosidase activity were sought by screening approximately 400,000 transformants.

Eighty-seven colonies that demonstrated β-galactosidase activity (i.e., colonies pale blue to dark blue on X-Gal plates) on the initial screen were isolated. Because the libraries were constructed using genomic DNA from a *GAL4* wild-type strain, plasmids containing the *GAL4* gene, or fragments thereof, are capable of activating transcription of the *GAL1-LacZ* reporter gene. These false positive colonies were eliminated by use of the polymerase chain reaction (PCR; White et al., (1989) Trends Genet. 5:185-189) with *GAL4* specific primers. The library plasmids from the remaining colonies were rescued and tested for specificity by retransforming them into the original strain with either: 1) the *GAL4-UPF1* fusion; 2) the *GAL4* DNA binding domain vector only; 3) an unrelated fusion, *GAL4-CEP1*; or 4) an unrelated fusion, *GAL4-LAM5* (Bartel et al., (1993) Biotechniques 14:920-924). Forty-two plasmids that yielded blue colonies only with *GAL4-UPF1* fusion plasmid-containing strains were characterized further by restriction mapping, Southern analysis, and partial DNA sequence analysis using standard techniques (see e.g., Sambrook et al., 1989, supra.

Blue colony formation occurred only when *NMD2* and UPF1 fusion plasmids were present in the same host strain. The *S. cerevisiae* tester strain GGY1::171 was co-transformed with the original library isolate pGAD2-*NMD2* and one of the following plasmids: 1) pMA424-*UPF1*, 2) pMA4242, 3) pMA424-*CEP1*, or 4) pMA424-*LAM5* (pMA424-*CEP1* was obtained from Richard Baker of the University of Massachusetts Medical Center, Worcester, MA; pMA424-*LAM5* was obtained from Stanley Fields and Paul Bartel of State University of New York, Stony Brook, N.Y.). Individual Leu^+ His^+ transformants were selected and streaked on

synthetic medium plates lacking histidine and leucine. β-galactosidase activity assays were performed by replica-plating the transformants onto SSX plates containing X-Gal. Cells were incubated at 30°C for 24-48 hours for development of blue color.

Southern blot analysis of the isolated plasmids was performed by first extracting total yeast genomic DNA according to the method of Holm et al. (1986) Gene 42:169-173. After restriction digestion, DNA was electrophoresed on 0.8% agarose gels, transferred and cross-linked to Zetaprobe membranes (BioRad, Richmond, CA) as described in Sambrook et al. (1989), supra. Filters were prehybridized for 2-3 hours at 42°C in 5X SSPE, 40% formamide, 5X Denhardt's solution, 0.1% SDS, and 4 mg/ml salmon sperm DNA. A radiolabeled NMD2 probe (1.2 kb *Cla*I-*Eco*RI fragment), generated by random priming, was added and filters were hybridized overnight at 42°C. Filters were washed twice in 1X SSC, 0.1% SDS at room temperature and once in 0.1X SSC, 0.1% SDS at 58°C before analyzing on a Betagen Blot Analyzer (Herrick, D. et al., (1991) Mol. Cell. Biol. 10:2269-2284).

DNA sequences were determined by the method of Sanger et al., (1978) Proc. Nat'l. Acad. Sci. USA 74:5463-5467. Overlapping fragments of the NMD2 gene were subcloned in Bluescript and sequenced by annealing oligonucleotide primers specific to the T3 or T7 promoter regions of the plasmid or by using oligonucleotide primers which annealed within the subcloned inserts.

Nine different genes were isolated by the following procedure. An *S. cerevisiae* genomic DNA library of Sau3A partial fragments constructed in YCp50 was used (Rose et al. (1987) Gene 60:237-243). Colony hybridization was performed as described in Sambrook et al., (1989), supra, using the same conditions described

for the genomic DNA Southern hybridization. Approximately three genomic equivalents were screened. Disruption of the *NMD2* gene was performed by transforming the diploid strain W303 (*MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100*) with a *Sac*I-*Sal*I fragment from Bs-*nmd2*::*HIS3* and selecting His$^+$ transformants (the *Sac*I and *Sal*I sites are in the polylinker of the Bluescript KS$^+$ cloning vector, Stratagene, La Jolla, CA; Rothstein (1991) "Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast.", in Methods in Enzymology 194: Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G. Fink, eds., Academic Press, pp. 281-301; Thomas and Rothstein (1989) Cell 56:619-630). The disruption event was confirmed by Southern analysis. Sporulation and tetrad analysis yielded haploid strains containing *nmd2*::*HIS3* disruptions.

Six of the isolated genes encoded putative Upf1p-interacting proteins because their activity in the assay was dependent on fusion to the *GAL4* activation domain. The remaining three genes did not require the presence of the *GAL4* activation domain, were likely to possess their own activation domains and nuclear localization signals and were not examined further.

Six genes were found to encode putative Upf1p-interacting proteins; two genes are identical to previously characterized yeast genes, i.e., *DBP2*, a gene encoding a putative RNA helicase with homology to the mammalian p68 RNA helicase (Iggo et al., (1991) Mol. Cell. Biol. 11:1326-1333). The other four have no apparent homologues in the available data bases. One of the genes, herein named *NMD2*, is characterized herein, and its uses for the production of heterologous proteins in yeast are disclosed.

Example 2: Molecular Cloning of the *NMD2* Gene

As defined by a qualitative β-galactosidase assay, Nmd2p showed a specific dependency on Upf1p in the two-hybrid system. Cells expressing a *GAL4* activation domain-*NMD2* fusion demonstrated strong β-galactosidase activity when simultaneously expressing a *GAL4* DNA-binding domain-*UPF1* fusion, but had no detectable β-galactosidase activity when co-transformed with plasmids encoding only the *GAL4* DNA-binding domain-*LAM5* fusion. Further evidence for the specificity of the interaction(s) was obtained by analyzing the effects of specific deletions within the *UPF1* portion of the *GAL4* DNA-binding domain-*UPF1* fusion. Deletions in all but one segment of the *UPF1* coding region eliminated Nmd2p-Upf1p interaction in the two-hybrid assay.

The *GAL4* activation domain-*NMD2* plasmid recovered in the two hybrid screen contained only a fragment of the *NMD2* gene. To isolate the entire gene, a 1.2 kb *Cla*I-*Eco*RI fragment downstream of the *GAL4* activation domain in the fusion plasmid was used to screen a yeast YCp50 genomic DNA library (Rose et al., (1987) supra). Two independent clones with identical restriction patterns were isolated. By restriction mapping, Southern analysis, and subsequent testing for complementation of an *NMD2* chromosomal deletion, the *NMD2* gene was localized to a 5.2 kb *Xba*I-*Sal*I DNA fragment as shown in Figs. 2A to 2C.

A restriction map of the *nmd2::HIS3* allele is shown in Fig. 2A. The *Xba*I-*Cla*I fragment of the *NMD2* gene, was deleted and replaced with the yeast *HIS3* gene. The left arrow in Fig. 2A represents the *HIS3* gene and indicates the direction of transcription. The right arrow of Fig. 2A represents the *NMD2* open reading frame.

A restriction map of the NMD2 gene is shown in Fig. 2B. The *NMD2* open reading frame and direction of

transcription are indicated by an open arrow interrupted by a stippled box that indicates the position of the intron. The box labeled probe indicates the DNA fragment used for screening the genomic DNA library. In Figs. 2A and 2B, the black box represents a segment from the cloning vector YCp50 and the restriction site abbreviations are: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Xb, *Xba*I.

To determine the regions of Nmd2p required for complementation of a disrupted nonsense mediated mRNA pathway in a *nmd2::HIS3* strain, deletion experiments were performed. In Fig. 2C, lines represent DNA fragments which were subcloned into an appropriate vector (such as pRS315). These constructs were transformed into the yeast strain HFY1300, or equivalent, which contains a partial chromosomal deletion of *NMD2* and lacks nonsense-mediated mRNA decay activity (see also, Figs. 3A and 3B). Total RNA was isolated from these transformants and Northern analysis was performed using a radiolabeled probe derived from the *CYH2* gene (He et al., (1993) Proc. Nat'l. Acad. Sci. USA 90:7034-7039). Complementing activity was scored by measuring the relative abundance of the *CYH2* pre-mRNA and mRNA in each strain. (+) and (-) indicate the ability or inability, respectively, to complement the *NMD2* chromosomal deletion, i.e., to restore the *CYH2* pre-mRNA to the marginally detectable levels characteristic of wild-type cells (He et al., (1993) Proc. Nat'l. Acad. Sci. USA 90:7034-7039).

To obtain a physical map position for the *NMD2* gene, the 1.7 kb *Xba*I-*Cla*I fragment was used to probe PrimeClone blots (American Type Culture Collection, Rockville, MD) containing characterized fragments of most of the *S. cerevisiae* genome (ATCC accession number 7155) known to lie on the right arm of chromosome VIII (Riles et al., (1993) Genetics 134:81-150). This fragment is

located between the *put2* and *CUP1* loci at a map position approximately 260 kb from the left telomere (Riles et al., (1993) supra).

Example 3: Determining the Primary Sequence of the *NMD2* Gene

The complete sequence of the *NMD2* gene was determined (SEQ ID NO:1). The *NMD2* coding region is 3267 nucleotides in length, encoding an acidic (predicted pI = 4.8) protein of 1089 amino acid residues (SEQ ID NO:2) with a predicted molecular weight of 127 kD. This interpretation of the *NMD2* sequence relies on the prediction of a 113-nucleotide intervening sequence that commences at position +7 and divides the gene into two exons (Figs. 1A-1C).

Four observations support the existence of this intron. First, the sequence contains all three of the standard consensus sequences expected of an intron (5' splice site [GUAUGU], branchpoint [UACUAAC], and 3' splice site [AG]) (Figs. 1A-1C). Second, as is true for most introns in yeast (Fink (1987) Cell 49:5-6), this intron is located at the 5' end of the NMD2 gene (six nucleotides downstream from the predicted initiator ATG; Figs. 1A-1C). Third, specific primer extension products were detected by using two different oligonucleotide primers complementary to mRNA sequences downstream of the predicted 3' splice site, but not by using a primer complementary to sequences within the intron. Finally, using the FLAG or c-*MYC* epitope tags (Hopp et al., (1988) Biotechnology 6:1204-1210; Prickett et al., (1989); Evan et al., (1985) Mol. Cell. Biol. 5:3610-3616) and epitope-specific monoclonal antibodies, the expression of a 127 kD polypeptide was detected when the FLAG or c-*MYC* sequences were inserted adjacent to the putative initiator ATG (FLAG-2-*NMD2* or c-*MYC*-*NMD2* alleles), but

not when the FLAG sequence was inserted adjacent to the second ATG (FLAG-1-NMD2 allele). The second ATG is located within the putative intron, 37 nucleotides downstream of the predicted intron branchpoint, and is in frame with the major downstream open reading frame but not with the first ATG. It is important to note that both the FLAG-1-*NMD2* and FLAG-2-*NMD2* alleles are functional in that they both show wild-type ability to complement a chromosomal deletion of *NMD2* (Fig. 2C). These results indicate that the FLAG-1 sequence inserted downstream of the second ATG has been removed by splicing out of the putative intron in the *NMD2* gene.

Analysis of the *NMD2* transcript was consistent with the predicted open reading frame. Northern analysis of total cellular RNA, using the *NMD2 Xba*I-*Cla*I fragment as a probe, identified a transcript of approximately 3.6 kb in size. Multiple transcription initiation sites were mapped to positions -56, -60, -64, and -67 using primer extension analysis (see e.g., Boorstein and Craig (1989) Meth. Enzymol. 180:347-369). A putative TATA box, required for most RNA polymerase II transcription (Struhl (1987) Cell. 49:295-297), lies at positions -219 to -213 in the *NMD2* promoter region and another regulatory element, an Abf1p binding consensus sequence (Della Seta et al., (1990) J. Biol. Chem. 265:15168-15175), is located within positions -198 to -186 (Figs. 1A-1C).

Structural features of the *NMD2* protein (Nmd2p; SEQ ID NO:2) inferred from the sequence analysis include a highly acidic internal fragment (36.8% aspartic acid and 25.6% glutamic acid) from residues 843 to 975 near the C-terminus and a possible bipartite nuclear localization signal at the N-terminus of the protein (i.e., within residues 26 to 29 and 42 to 46) (Figs. 1A-1C; Dingwall and Laskey, (1991) supra).

Comparison of the Nmd2p sequence with those in the Swissprot and Pir protein sequence databases using the FASTA or TFASTA comparison programs (Devereux et al., (1984) supra) did not reveal any extensive identity with known protein sequences. However, three domains of Nmd2p have substantial similarity to regions of other proteins. The first domain, spanning Nmd2p amino acids 1 to 390, has 17.7% sequence identity and 47% similarity with translational elongation factor 2 (Eft1p and Eft2p) from *S. cerevisiae* (Perentesis et al., (1992) J. Biol. Chem. 267:1190-1197). The second domain, from amino acids 400 to 810 in Nmd2p, shares 19.5% sequence identity and 42.6% similarity with the *S. cerevisiae* mitochondrial RNase P protein Rpm2p (Dang and Martin (1993) J. Biol. Chem. 268:19791-19796).

The third domain, encompassing the acidic stretch from amino acids 820 to 940, has 34% sequence identity and 63.2% similarity with human and mouse nucleoproteins (Lapeyre et al., (1987) Proc. Natl. Acad. Sci. 84:1472-1476; Bourbon et al., (1988) J. Mol. Biol. 200:27-638) and 34% identity and 65% similarity to the mammalian polymerase I transcriptional factors hUBF and mUBF (Jantzen et al., (1990) Nature 344:830-836; Hisatake et al., (1991) Nucleic Acids Res. 19:4631-4637). In hUBF and mUBF this domain has been shown to be important for interaction with other proteins (Jantzen et al., (1990) supra) and, as described below, is also true for Nmd2p.

Example 4: *NMD2* Disruption Does Not Affect Cell Viability and Selectively Stabilizes Nonsense-containing mRNAs

A *NMD2* gene disruption experiment was performed to assess the cellular requirement for Nmd2p. The *nmd2::HIS3* disruption described in Fig. 2A was constructed. Plasmid Bs-*nmd2::HIS3* encodes the same *NMD2* disruption and contains a 0.6 kb *Cla*I-*Xba*I fragment in

the 5'-end of *NMD2*, a 1.7 kb *Xba*I-*Cla*I fragment of *HIS3* and a 1.2 kb *Cla*I-*Eco*RI fragment in the *NMD2* coding region in Bluescript. A *Sac*I-*Sal*I fragment carrying the *nmd2::HIS3* allele was isolated from plasmid Bs-*nmd2::HIS3* and used to transform the yeast diploid strain W303 for homologous recombination into one of the *NMD2* alleles. His^+ transformants were sporulated and tetrads were individually dissected. Four viable spores were obtained from each tetrad analyzed. Genomic DNAs from parental diploid and progeny haploid strains were isolated, digested with *Eco*RI. Confirmation of integration is shown by the Southern analysis of Fig. 3A in which lane P1 contains DNA isolated from the homozygous *NMD2/NMD2* diploid strain W303; lane P2 contains DNA isolated from a diploid *nmd2::HIS3/NMD2* His^+ transformant of W303 (HFY1000); and lanes 1A to 1D contain DNA isolated from the progeny of four viable spores dissected from the same tetrad represent the wild-type and disrupted alleles of *NMD2*, respectively. Other bands in the figure are not specific to *NMD2*.

Haploid strains containing the *nmd2::HIS3* disruption were compared to isogenic *NMD2* strains for their ability to grow on different carbon sources (glucose, galactose, and glycerol) at temperatures ranging from 18°C to 37°C and no differences in growth rates were detected between mutant and wild-type strains. These data indicate that *NMD2* is non-essential for cell viability. Since disruption of the *NMD2* gene was not lethal, the activities of the nonsense-mediated mRNA decay pathway in both *NMD2* and *nmd2::HIS3* strains were compared.

The following method was used to analyze transcript stability in strains having an *NMD2* disruption, and is useful to one of ordinary skill in the art for analyzing the stability of any transcript of

interest. Yeast centromere plasmids carrying six different *PGK1* nonsense alleles were constructed previously (Peltz et al., (1993) supra). These plasmids were transformed into *NMD2* and *nmd2::HIS3* strains and the abundance of *PGK1* nonsense-containing mRNAs was assessed by Northern analysis as shown in Fig. 3B. Disruption of the *NMD2* gene stabilizes *PGK1* mRNAs containing early nonsense mutations. Isogenic *NMD2* and *nmd2::HIS3* haploid yeast strains harboring different nonsense-containing *PGK1* alleles (HFY1201 to HFY1206 and HFY1301 to HFY1306) were constructed by transforming HFY1200 and HFY1300 with each of the six plasmids harboring the nonsense-containing *PGK1* alleles described previously (Peltz et al., (1993) Genes & Dev. 7:1737-1754) and herein incorporated by reference.

Total RNA was isolated from these strains and analyzed by Northern blotting using a radiolabeled oligonucleotide probe complementary to the tag sequence located in the 3' untranslated region of *PGK1* nonsense-containing mRNAs (Peltz et al., (1993) Genes & Dev. supra). The location of the nonsense mutation in each *PGK1* transcript is presented as a percentage of the *PGK1* protein-coding region that is translated before the mutation is encountered (Peltz et al., (1993) supra).

Decay rates of mRNA were measured as previously described (Herrick et al., (1990) supra; Parker et al., (1991) Meth. Enzymol. 194:415-423; Peltz et al., (1993) supra). For measurement of mRNA abundance, yeast cells (20 ml) were grown to OD_{600}=0.5-0.7 at 24°C for 30 minutes. An aliquot (2 ml) of concentrated cell culture was collected and frozen quickly on dry ice. Total yeast RNA was isolated as described previously (Herrick et al., (1991) supra). For both decay rate measurements and abundance measurements equal amounts (usually 20 μg) of total RNA from each sample were analyzed by Northern

blotting, generally using probes labeled in random priming reactions (see, e.g., Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Hybridization conditions for such blots were as described for genomic Southern hybridization. When oligonucleotide probes were used, the hybridization conditions were those described by Peltz et al. (1993) supra. Northern blots were quantitated with a Betagen Blot Analyzer (Herrick et al., (1990) supra).

Nonsense mutations in the 5′ two-thirds of the *PGK1* coding region reduced the abundance of the corresponding mRNAs 5- to 20-fold (Peltz et al., (1993) supra). The abundance of *PGK1* mRNAs with nonsense mutations in the downstream third of the coding region is unaffected. Disruption of the *NMD2* gene restored wild-type levels to all four of the *PGK1* transcripts normally subject to nonsense-mediated mRNA decay (Fig. 3B). As a control, the abundance of the wild-type *PGK1* and *ACT1* mRNAs, and the half-life of the MATα1 mRNA in the same cells, was found to be unaffected by the *nmd2::HIS3* disruption.

Northern analysis was also used to measure the relative abundance of the *CYH2*, *RP51B*, and *MER2* pre-mRNAs in *NMD2*. As shown in Fig. 3C, decay rates of *CYH2* pre-mRNA and mRNA were determined by Northern analysis of RNAs isolated at different time points after transcription was inhibited by shifting cultures of isogenic *NMD2* (HFY2206) and *nmd2* (HFY2106) strains to 36°C. Samples were taken for 36 minutes and the blot was hybridized with a radiolabeled *CYH2* DNA probe. To construct strains HFY2206 and HFY2106, strain HFY2000 was produced by integrative transformation; selected and tested to contain the temperature-sensitive *rpb*1-1 allele. Strain HFY2000 was transformed with pRS315 (or

similar yeast shuttle plasmid; (Sikorski and Hieter, (1989) Genetics 122:19-27) or pRS315-*NMD2*(X-S) (containing a 5.2 kb *Xba*I-*Sal*I fragment of *NMD2* in pRS315) and a plasmid harboring a *PGK1* allele with a nonsense mutation at the *Bgl*II site (Peltz et al., (1993) supra). The abundance of the inefficiently spliced *CYH2* and *RP51B* pre-mRNAs, and the *MER2* pre-mRNA (whose splicing is regulated by *MER1*; Engebracht et al., 1991) was markedly increased in strains carrying the *nmd2::HIS3* disruption. Disruption of the *NMD2* gene reduces the decay rate of the *CYH2* pre-mRNA approximately four-fold, i.e., from a half-life of 1.5 min to a half-life of 6.0 min without a concomitant effect on the half-life of the *CYH2* mRNA (Fig. 3C). These results are equivalent to those obtained in *UPF1* knockout strains (He et al., (1993) supra) indicating that Nmd2p is a Upf1p-interacting protein and that *NMD2* is a novel component of the nonsense-mediated mRNA decay pathway.

Example 5: Overexpression of Truncated Nmd2p in the Cytoplasm Results in a Dominant-negative Nonsense-mediated mRNA Decay Phenotype

The region of Nmd2p that interacts with Upf1p was determined by generating 5′ and 3′ deletions of the original *NMD2* fragment, fusing them in-frame to the *GAL4* activation domain, and assaying the resultant constructs for interaction with Upf1p using the two-hybrid system. Fusions encoding either 237 or 477 amino acids from the amino-terminus of the original fragment demonstrated no detectable β-galactosidase activity. However, fusions encoding either 526 or 286 amino acids from the carboxyl-terminus of the original fragment did demonstrate detectable β-galactosidase activity. These results indicate that the acidic C-terminal domain of Nmd2p interacts with Upf1p.

The identification of Nmd2p as a Upf1p-interacting protein in a two-hybrid screen and the observation that disruption of the *NMD2* gene yielded a nonsense-mediated mRNA decay phenotype equivalent to that obtained in strains harboring *upf1* mutations suggests that Upf1p and Nmd2p interact with each other *in vivo* and that they perform different functions in the same decay pathway. This conclusion is strengthened by the finding that double mutants in which both the *UPF1* and *NMD2* gene products are functionally absent produce strains that have essentially identical phenotypes with regard to the half-lives of test mRNA transcripts such as *CYH2* pre-mRNA. Thus, Upf1p and Nmd2p must function in closely related steps of the nonsense-mediated mRNA decay pathway.

A truncated form of Nmd2p was expressed in both the nucleus and cytoplasm and activity was functionally localized within the cell to the cytoplasm. The original *GAL4* activation domain-*NMD2* fusion plasmid encodes 764 amino acids of the C-terminal segment of Nmd2p (SEQ ID NO:4). Transcription of this *GAL4*-activation domain-*NMD2* fusion was driven by a cryptic promoter in the *ADH1* terminator present in the vector and the fusion protein was targeted to the nucleus by the SV40 T antigen nuclear localization signal (Chien et al., (1991) supra. The 6.0 kb *Hin*dIII fragment encoding this fusion protein was also subcloned into pGAD2F so that transcription of the fusion protein was driven by the more potent *ADH1* promoter. Since the SV40 T antigen nuclear localization signal (NLS) of the fusion protein is in a 36 bp *Eco*RI fragment (Benton et al., (1990) Mol. Cell. Biol. 10:353-360, we also generated deletions of the NLS in the respective constructs. Plasmids expressing the different fusion proteins were transformed into the haploid strain HFY1200 which is wild-type for both UPF1 and NMD2. HFY1200 was

derived from W303 by standard techniques (see, e.g., Rothstein, R. (1991) "Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast.", in Methods in Enzymology 194: Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G. Fink, eds., Academic Press, pp. 281-301). Control experiments, using the two hybrid assay, showed that when *NMD2* plasmids lacking the T antigen NLS were co-transformed with the original plasmid encoding the *GAL4* DNA binding domain-*UPF1* fusion no β-galactosidase activity was detectable, i.e., nuclear localization had been eliminated. Total RNA was isolated from transformants and Northern analysis was performed using a fragment of the *CYH2* gene as a probe.

The Northern analysis results depicted in Fig. 4A show that a double mutant containing both *upf1::URA3* and *nmd2::HIS3* disruptions is phenotypically identical to either *upf1* or *nmd2* single mutants since the *CYH2* pre-mRNA is stabilized in cells containing these disruptions. Total RNAs were isolated from each of the following strains: HFY3002 (*UPF1/NMD2*); HFY3005 (*upf1Δ/NMD2*); HFY3008 (*UPF1/nmd2Δ*) and HFY3001 (*upf1Δ/nmd2Δ*) (see Table 2). RNAs were analyzed by Northern blotting using a radiolabeled *CYH2* fragment as probe.

TABLE 2 - Yeast Strains

STRAIN	GENOTYPE
HFY1000	*MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 nmd2::HIS3/NMD2*
HFY1100	*MATα ade2-1 his3-11,15 leu2-3,112trp1-1 ura3-1 can1-100 NMD2*
HFY1200	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 NMD2*
HFY1300	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 nmd2::HIS3*
HFY1400	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 nmd2::HIS3*
HFY1201	*Same as HFY1200 but containing [pRIPPGKH2 (3) UAG]*
HFY1202	*Same as HFY1200 but containing [pRIPPGKAsp UAG]*
HFY1203	*Same as HFY1200 but containing [pRIPPGKH2 (2) UAG]*
HFY1204	*Same as HFY1200 but containing [pRIPPGKH2 (1) UAG]*
HFY1205	*Same as HFY1200 but containing [pRIPPGKXba UAG]*
HFY1206	*Same as HFY1200 but containing [pRIPPGKBgl UAG]*
HFY1301	*Same as HFY1300 but containing [pRIPPGKH2 (3) UAG]*
HFY1302	*Same as HFY1300 but containing [pRIPPGKAsp UAG]*
HFY1303	*Same as HFY1300 but containing [pRIPPGKH2 (2) UAG]*
HFY1304	*Same as HFY1300 but containing [pRIPPGKH2 (1) UAG]*
HFY1305	*Same as HFY1300 but containing [pRIPPGKXba UAG]*
HFY1306	*Same as HFY1300 but containing [pRIPPGKBgl UAG]*
HFY2000	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpb1-1 nmd2::HIS3*
HFY2106	*Same as HFY2000 but containing [pRS315] [pRIPPGKBgl UAG]*
HFY2206	*Same as HFY2000 but containing [pRS315-NMD2 (X-S)] [pRIPPGKBgl UAG]*
HFY3000	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 nmd2::HIS3 upf1::URA3*
HFY3001	*Same as HFY3000 but containing [pRS315] [pRS314]*
HFY3002	*Same as HFY3000 but containing [pRS315-NMD2 (X-S)] [pRS314-UPF1]*
HFY3005	*Same as HFY2000 but containing [pR315- NMD2 (X-S)] [pRS314]*
HFY3008	Same as HFY2000 but containing [pRS315] [pRS314-*UPF1]*

The strains listed in Table 2 were prepared in this study. See Peltz et al. (1993), supra, for a description of the "pRIPPGK__" plasmids listed above.

Overexpression of truncated Nmd2p in the cytoplasm results in a dominant-negative nonsense-mediated mRNA decay phenotype as shown in Fig. 4B. The yeast strain HFY1200 which is wild-type for both *UPF1* and *NMD2* was transformed with pGAD2F-*NMD2*-ADHt, pGAD2F-*NMD2*-ADHp, pGAD2F, pGAD2F-*NMD2*-ADHt-ΔNLS, pGAD2F-*NMD2*-ADHp-ΔNLS, respectively (see Table 3). Total RNA was isolated from these transformants and analyzed by Northern blotting using a *CYH2* DNA fragment as probe. Lane 1 contained RNA isolated from HFY1300 (control); RNA in other lanes was from transformants of HFY1200 harboring the following plasmids; lane 2, pGAD2F-*NMD2*-ADHt; lane 3, pGAD2F-*NMD2*-ADHp; lane 4, pGAD2F; lane 5, pGAD2F-*NMD2*-ADHt-ΔNLS; lane 6, pGAD2F-*NMD2*-ADHp-ΔNLS. Overexpression of truncated *NMD2* fusion protein localized to the nucleus had no effect on the accumulation of the *CYH2* pre-mRNA (Fig. 4B, lanes 2 and 3). Expression of the cytoplasmically localized fusion protein caused an accumulation of *CYH2* pre-mRNA in a dosage dependent manner, i.e., expression of the fusion protein from the stronger promoter led to a greater accumulation of the *CYH2* pre-mRNA than expression from the weaker promoter (Fig. 4B, lanes 5 and 6). This result establishes that over-expression of a truncated form of the Nmd2p C-terminus (i.e., containing up to 764 amino acids from the C-terminus (SEQ ID NO:4)) results in inhibition of the nonsense-mediated mRNA decay pathway. Shorter C-terminal fragments of Nmd2p are included in the invention as they are readily obtained by screening for inhibiting activity by the two-hybrid screening method coupled with analysis of heterologous transcript stability in the presence of overexpressed amounts of the fragment in the host strain.

TABLE 3 - Plasmids

PLASMIDS	RELEVANT YEAST SEQUENCES
pGAD2F	*GAL4* activation domain-containing plasmid with 2μ and *LEU2* selection markers (Chien, C.-T. et al. (1991) PNAS 88:9578-9582).
pGAD2F-*NMD2*-ADHp	6.0-kb *HindIII* fragment from pGAD2-*NMD2* replaced the 0.6-kb *HindIII-HindIII* fragment of pGAD2F such that the expression of the *GAL4* activation domain -*NMD2* fusion was driven by the *ADH1* promoter.
pGAD2F-*NMD2*-ADHt	6.0-kb *HindIII* fragment from pGAD2-*NMD2* replaced the 0.6-kb *HindIII-HindIII* fragment of pGAD2F such that the expression of the *GAL4* activation domain -*NMD2* fusion was driven by the *ADH1* terminator.
pGAD2F-*NMD2*-ADHp-ΔNLS	Same as pGAD2F-*NMD2*-ADHp except that the SV40 nuclear localization signal of the fusion protein was deleted.
pGAD2F-*NMD2*-ADHt-ΔNLS	Same as pGAD2F-*NMD2*-ADHt except that the SV40 nuclear localization signal of the fusion protein was deleted.

Example 6: Expression of *NMD2* Antisense Transcript Inhibits the Nonsense-Mediated mRNA Decay Pathway

Nonsense-mediated mRNA decay pathway function of a host cell (i.e., a prokaryotic or eukaryotic cell such as a yeast cell) is reduced or inhibited by providing within the cell a portion of the antisense strand of the *NMD2* gene introduced into cells in which *NMD2* is transcribed. The antisense oligonucleotide (either RNA or DNA) can be directly introduced into the cells in a form that is capable of binding to the *NMD2* sense transcripts. Alternatively, a vector containing sequence which, once within the host cells, is transcribed into the appropriate antisense mRNA, can be the species administered to the cells. An antisense nucleic acid that hybridizes to the mRNA of the target gene can

decrease or inhibit production of the polypeptide product encoded by the gene by forming a double-stranded segment on the normally single-stranded mRNA transcript, thereby interfering with translation. It may be preferable to select sequences for antisense applications that do not contain nonsense codons as these may stimulate rather than inhibit the nonsense-mediated mRNA decay pathway.

A DNA sequence, such as a full or partial sequence of the *NMD2* gene, is expressed as an antisense transcript. The sequence can be operably linked to appropriate expression control sequences and introduced into host cells by standard techniques well known to those of ordinary skill in the art. An effective amount of the expressed antisense transcript is produced such that translation of the *NMD2* sense mRNA transcript is inhibited. By an equivalent method, UPF1 mRNA antisense transcript or a fragment thereof which binds to the UPF1 sense transcript, inhibiting translation and thereby, inhibiting the nonsense-mediated mRNA pathway. Antisense transcript production can be constitutive or controlled, as desired, according to the transcription regulatory sequences operably linked to the *NMD2* or UPF1 DNA sequences for the production of antisense transcript.

Inhibition of the nonsense-mediated mRNA pathway using antisense transcripts to inhibit translation of a protein of the pathway (such as *NMD2* or *UPF1*) is useful to enhance the stability of a nonsense codon-containing transcript which encodes a heterologous polypeptide to be produced in yeast cells or to enhance the production of a mutated endogenous polypeptides useful to the host cell or host organism.

Antisense transcripts are also useful for treating genetic disorders involving a nonsense mutation. For example, using gene therapy methods known to those in the art, a vector able to express antisense transcripts for a

gene in the nonsense-mediated mRNA decay pathway (e.g., *NMD2* or *UPF1*) is introduced into a patient harboring a disease-causing nonsense mutation.

Example 7: Production of Heterologous Protein or Polypeptide in a Yeast Cell Inhibited in the Nonsense-Mediated mRNA Pathway

A protein or polypeptide of interest is produced by providing an expression vector encoding a gene for a heterologous protein. The expressed transcript of the gene encodes a nonsense codon in a transcript destabilizing 5′ portion of the transcript such that the transcript is at least 2 fold less stable in a wild-type strain than in a nonsense-mediated mRNA decay-inhibited host strain. Nonsense-mediated mRNA decay is inhibited by 1) mutating the *NMD2* gene such that no functional Nmd2p is produced; 2) overexpressing a C-terminal fragment of Nmd2p such that the fragment binds to UPF1 inhibiting its function; or 3) expressing sufficient *NMD2* or UPF1 antisense transcript to hybridize to *NMD2* or UPF1 sense transcript preventing its translation into functional Nmd2p or Upf1p, respectively. All of these methods can be carried out by standard procedures.

If it is desired that an amino acid be substituted at the nonsense codon position, then the host strain used is also an amino acid substitution suppressor strain. The suppressor strain is chosen such that a specific amino acid (dictated by the type of suppressor mutation in the host strain) is substituted at the nonsense codon. The substituted amino acid can be an amino acid encoded by the natural codon at that site. The substituted amino acid can be different from the naturally encoded amino acid if it is desired to test the effect of that amino acid on the conformation or activity of the encoded protein.

If the heterologous protein to be expressed is toxic to the host cell, inhibition of the nonsense-mediated mRNA decay pathway can be controlled by the inducible expression of, for example, Nmd2p C-terminal fragment or *NMD2* antisense transcript. Controllable inhibition of the decay pathway allows transcript stabilization and translation a point in the host yeast cell culture growth such that maximum production of the toxic protein occurs prior to the death of the host cells.

Following inhibition of the nonsense-mediated mRNA pathway and translation of the stabilized nonsense codon-containing transcript into the desired heterologous protein or protein fragment, the protein or fragment is isolated from the yeast host cells by standard protein purification methods.

Example 8: Production of Antibody to Nmd2p or a C-terminal Fragment of Nmd2p

Nmd2p or Nmd2p C-terminal fragment polypeptide of the invention can be produced by first transforming a suitable host cell with the entire NMD2 gene (for the production of Nmd2p) or with a partial NMD2 sequence (encoding the C-terminal part of Nmd2p), respectively, cloned into a suitable expression vehicle followed by expression of the desired protein or polypeptide.

Those of ordinary skill in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the protein or polypeptide. The precise host cell used is not critical to the invention. The polypeptide can be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*). The method of transformation of the cells and the choice of expression vehicle will depend on the host system selected. Methods described herein provide sufficient guidance to

successfully carry out the production, purification and identification of Nmd2p or THE Nmd2p C-terminal fragment.

Once the Nmd2p or Nmd2p C-terminal fragment (or fragment or analog thereof) is expressed, it is isolated, e.g., using immunoaffinity chromatography. In one example, an anti-Nmd2p or anti-(Nmd2p C-terminal fragment) antibody can be attached to a column and used to isolate Nmd2p or Nmd2p C-terminal fragment, respectively. Lysis and fractionation of Nmd2p or Nmd2p C-terminal fragment-containing host cells prior to affinity chromatography can be performed by standard methods. Once isolated, the recombinant protein can, if desired, be further purified, e.g, by high performance liquid chromatography (see e.g., Fisher, Laboratory Techniques In Biochemistry and Molecular Biology, eds., Work and Burdon, Elsevier, (1980).

Nmd2p or fragments thereof, particularly short fragments which inhibit the nonsense-mediated mRNA decay pathway, can also be produced by chemical synthesis, by standard solution or solid phase peptide synthesis techniques.

Substantially pure Nmd2p or Nmd2p C-terminal fragment can be used to raise antibodies. The antibodies are useful for screening, by Western blot analysis, host stains overexpressing Nmd2p or Nmd2p C-terminal fragment, thereby identifying candidate strains which produce a desired amount of Nmd2p or Nmd2p C-terminal fragment.

Antibodies directed to the polypeptide or interest, Nmd2p or NMd2p C-terminal fragment, are produced as follows. Peptides corresponding to all or part of the polypeptide of interest are produced using a peptide synthesizer by standard techniques, or are isolated and purified as described above. The peptides are coupled to KLH with M-maleimide benzoic acid N-hydroxysuccinimide ester. The KLH-peptide is mixed with

Freund's adjuvant and injected into animals, e.g., guinea pigs or goats, to produce polyclonal antibodies.

Monoclonal antibodies can be prepared using the polypeptide of interest described above and standard hybridoma technology (see, e.g., Kohler et al., Nature (1975) 256: 495; Kohler et al., Eur. J. Immunol. (1976) 6:292, Kohler et al., Eur. J. Immunol. (1976) 6:511; Hammerling et al., in Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, (1981), which are incorporated herein by reference). Antibodies are purified by peptide antigen affinity chromatography.

Once produced, antibodies are tested for specific Nmd2p or Nmd2p C-terminal fragment binding by Western blot or immunoprecipitation analysis by standard techniques.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci.* USA, 81:6851, 1984; Neuberger et al., *Nature*, 312-604, 1984; Takeda et al., *Nature*, 314:452, 1984) can be used. These methods involve splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Example 9: Identification of Murine Homologs of Yeast *NMD2*

Several different approaches to identifying murine NMD2 homologs can be utilized.

In one approach, comparative genomics are used to identify murine homologs of NMD2. The sequence of the

complete yeast NMD2 protein is compared to existing databases of random cDNA sequences (Bassett, D.J., et al., *Trends Genet.* 1:4372-373, 1995). Fragments with significant homology to NMD2 are used as nucleic acid probes in subsequent screens of murine genomic DNA and cDNA libraries as described herein. Full-length genes and cDNAs having substantial homology to NMD2 are then further characterized as described herein.

Two-hybrid screens can also be used to identify murine homologs of NMD2. Mouse genes encloding proteins that interact with yeast UPF1 or UPF3 proteins (He et al., *Mol. Cell. Biol.* 17:1580-1594, 1997), or human homologs of the yeast UPF1 protein (Perlick et al., Proc. Natl. Acad. Sci. USA 93:10928-10932, 1996; Applequist et al., Nucleic Acids Res. 25:814-821, 1997), are identified using the two-hybrid method (Fields and Song, Nature 340:245-246, 1989; Chien et al., Proc. Natl. Acad. Sci. USA 88:9578-9582, 1991; Fields and Sternglanz, Trends Genet. 10:286-292, 1994; Bartel and Fields, Methods Enzymol. 254:241-263, 1995). DNA encoding the UPF protein is cloned and expressed from plasmids harboring GAL4 or lexA DNA-binding domains and co-transformed into cells harboring lacZ and HIS3 reporter constructs along with libraries of cDNAs that have been cloned into plasmids harboring the GAL4 activation domain. Libraries used for such co-transformation include those made from B-cells and T-cells since such cells may have high activities of the nonsense-mediated mRNA decay pathway.

Another method for identifying murine homologs of NMD2 utilize complementation of yeast NMD2 mutants. Yeast UPF1 mutants incapable of nonsence-mediated mRNA decay suppress the growth defects of cells harboring nonsense mutations in the LEU2 or TYR7 genes (Leeds et al., Mol. Cell. Biol. 12:2165, 1992; Peltz et al., Prog. Nucleic Acids Res. Molec. Biol. 47:271-298, 1994).

Comparable effects are observed with NMD2 mutants, or in cells harboring the NMD2 dominant-negative fragment. In all of these cases, cells with the leu2-3 or tyr7-1 nonsense mutations fail to grow in the absence of leucine or tyrosine, respectively, if they are wild-type for nonsense-mediated mRNA decay and will grow in the absence of these amino acids if they have defects in the nonsense-mediated mRNA decay pathway. Therefore, NMD2 mutants, or cells harboring the NMD2 dominant-negative fragment, are transformed with mammalian cDNA libraries cloned into yeast plasmid vectors. The transformants are analyzed for restoration of the wild-type pattern, i.e., failure to grow in the absence of leucine or tyrosine.

Alternatively, the transformed cells are screened for their ability to grow in the presence of 100 μg/ml of the growth inhibitor canavanine. This drug enters cells via arginine permease, a protein encoded by the CAN1 gene. Cells harboring the can1-nonsense mutation are resistant to 100 μg/ml canavanine if they are wild-type for nonsense-mediated mRNA decay, but sensitive to it if they also harbor an NMD2 mutation. Hence, restoration of NMD2 function by exogenous DNA is assessed by plating cells on canavanine. Plasmids are isolated from the cells surviving on canavanine, the plasmids are sequenced, and the sequences analyzed to confirm murine sequence that complements mutant nmd2 (e.g., the cell is not a revertant).

PCR with degernate oligonucleotides is also a method of identifying murine NMD2 homologs. Homologs of the NMD2 gene are identified in other, non-murine, species are compared to identify specific regions with a high degree of homology. These regions of high homology are selected for the design of PCR primers that maximize possible base-pairing with heterlogous genes. Construction of such primers involves the use of

oligonucleotide mixtures that account for degeneracy in the genetic code, i.e., allow for the possible base changes in murine NMD2 genes that do not affect the amino acid sequence of the NMD2 protein. Such primers are used to amplify and clone possible NMD2 gene fragments from mouse DNA. The latter are sequenced and those encoding protein fragments with high degrees of homology to fragments of yeast NMD2 protein are used as nucleic acid probes in subsequent screens of murine genomic DNA and cDNA libraries. Full-length genes and cDNAs having substantial homology to yeast NMD2 are identified in these screens.

Example 10: Identification of Human Homologs of Yeast *NMD2*

The human homolog of the yeast *NMD2* gene is useful for the elucidation of the biochemical pathways of nonsense-mediated mRNA decay in mammals as well as for the development of treatments for genetic disorder involving nonsense mutations. Several approaches can be used to isolate human *NMD2* genes including a two-hybrid screen, complementation of yeast NMD2 mutants by expression libraries of cloned human cDNAs, polymerase chain reactions (PCR) primed with degenerate oligonucleotides, low stringency hybridization screens of human libraries with the yeast NMD2 gene, and database screens for homologous sequences. The human *NMD2* gene can also be identified by appropriate application of the above methods based on homology with the mouse *NMD2* gene homolog.

Methods of screening for and identifying human homologs of NMD2 are described above (e.g., Example 8). In addition, the murine homolog of NMD2 can be used instead of the yeast Nmd2 sequence to probe a human cDNA or genomic DNA library for homologous sequences.

To produce the human NMD2 gene product (e.g., human Nmd2p) the human *NMD2* gene is placed in an expression vector and the gene expressed in an appropriate cell type. Human *Nmd2p* is isolated from such cell lines using methods known to those in the art, and used in the assays described below.

Example 11: Methods of Screening for Molecules that Inhibit the Nonsense-mediated mRNA Decay Pathway

The following assays are designed to identify compounds that are effective inhibitors of the nonsense-mediated mRNA decay pathway. Such inhibitors may act by, but are not limited to, binding to an Ndm2p (e.g., from yeast, mouse or human), binding to intracellular proteins that bind to an Nmd2p, compounds that interfere with the interaction between Nmd2p and nonsense mutation-containing mRNA, compounds that modulate the activity of an NMD2 gene, or modulate the expression of an NMD2 gene or an Nmd2p.

Assays can also be used to identify molecules that bind to nonsense-mediated mRNA decay pathway gene regulatory sequences (e.g., promoter sequences), thus modulating gene expression. See e.g., Platt, 1994. *J. Biol. Chem.* 269:28558-28562, incorporated herein in its entirety.

The compounds which may be screened by the methods described herein include, but are not limited to, peptides and other organic compounds (e.g., peptidomimetics) that bind to a nonsense-mediated mRNA decay pathway protein (e.g., that bind ton an Nmd2p), or inhibit its activity in any way.

Such compounds may include, but ar not limited to, peptides; for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam et al., 1991. *Nature* 354:82-94; Houghten et

al., 1991. *Nature* 354:84-86), and combinatorial chemistry-derived molecular libraries made of D-and/or L-amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see e.g., Songyang et al., 1993. *Cell* 72:767-778), and small organic or inorganic molecules.

Organic molecules are screened to identify candidate molecules that affect expression of a nonsense-mediated mRNA decay (e.g., NMD2) gene or some other gene involved in the nonsense-mediated mRNA decay pathway (e.g., by interacting with the regulatory region or transcription factors of a gene). Compounds are also screened to identify ones that affect the activity of such proteins, (e.g., by inhibiting Nmd2p activity) or the activity of a molecule involved in the regulation of, for example, Nmd2p.

Computer modelling or searching technologies are used to identify compounds, or identify modifications of compounds that modulate the expression or activity of a nonsense-mediated mRNA decay protein. For example, compounds likely to interact with the active site of a protein (e.g., Nmd2p) are identified. The active site of an Nmd2p molecule can be identified using methods known in the art including, for example, analysis of the amino acid sequence of a molecule, from a study of complexes of Nmd2p, with its native ligand (e.g., Upf1p). Chemical or X-ray crystallographic methods can be used to identify the active site of Nmd12p by the location of a bound ligand such as Upf1p.

The three-dimensional structure of the active site is determined. This can be done using known methods, including X-ray crystallography which may be used to determine a complete molecular structure. Solid or liquid phase NMR can be used to determine certain intra-

molecular distances. Other methods of structural analysis can be used to determine partial or complete geometrical structures. Geometric structure can be determined with an Nmd2p bound to a natural (e.g., Upf1p) or artificial ligand which may provide a more accurate active site structure determination.

Computer-based numerical modelling can be used to complete an incomplete or insufficiently accurate structure. Modelling methods that may be used are, for example, parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups are necessary, and can be selected from force field known in physical chemistry. Information on incomplete or less accurate structures determined as above can be incorporated as constraints on the structures computed by these modeling methods.

Having determined the structure of the active site of a nonsense-mediate mRNA protein (.e.g, Nmd2p, either experimentally, by modeling, or by a combination of methods, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. The compounds identified in such a search are those that have structures that match the active site structure, fit into the active site, or interact with groups defining the active site. The compounds identified by the search are potential nonsense-mediated mRNA decay pathway modulating compounds.

These methods may also be used to identify improved modulating compounds from an already known modulating compound or ligands. The structure of the

known compound is modified and effects are determined using experimental and computer modelling methods as described above. The altered structure may be compared to the active site structure of a nonsense-mediated mRNA decay protein (e.g., an Nmd2p) to determine or predict how a particular modification to the ligand or modulating compound will affect its interaction with that protein. Systematic variations in composition, such as by varying side groups, can be evaluated to obtain modified modulating compounds or ligands of preferred specificity or activity.

Other experimental and computer modeling methods useful to identify modulating compounds based on identification of the active sites of a nonsense-mediated mRNA decay protein and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modelling systems are the QUANTA programs, e.g., CHARMm, MCSS/HOOK, and X-LIGAND, (Molecular Simulations, Inc., San Diego, CA). QUANTA analyzes the construction, graphic modelling, and analysis of molecular structure. CHARMm analyzes energy minimization and molecular dynamics functions. MCSS/HOOK characterizes the ability of an active site to bind a ligand using energetics calculated via CHARMm. X-LIGAND fits ligand molecules to electron density of protein-ligand complexes. It also allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

Articles reviewing computer modelling of compounds interacting with specific protein can provide additional guidance. For example, see Rotivinen et al., 1988 *Acta Pharmaceutical Fennica* 97:159-166: Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly and Rossmann, 1989, *Ann. Rev. Pharmacol. Toxicol.* 29:111-122; Perry and Davies.

OSAR Quantitative Structure -Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc., 1989); Lewis and Dean, 1989, *Proc. R. Soc. Lond.* 236:125-140, 141-152; and, regarding a model receptor for nucleic acid components, Askew et al., *Am. J. Chem. Soc.* 111:1082-1090. Computer programs designed to screen and depict chemicals are available from companies such as MSI (*supra*), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Gainesville, FL).

These applications are largely designed for drugs specific to particular proteins; however, they can be adapted to the design of drugs specific to identified regions of DNA or RNA. Chemical libraries that can be used in the protocols described herein include those available, e.g., from ArQule, Inc. (Medford, MA) and Oncogene Science, Inc. (Uniondale, NY).

In addition to designing and generating compounds that alter binding, as described above, libraries of known compounds, including natural products, synthetic chemicals, and biologically active materials including peptides, can be screened for compounds that are inhibitors or activators of the nonsense-mediated mRNA decay pathway.

Compounds identified by methods described above can be used, for example, for elaborating the biological function of nonsense-mediated mRNA decay pathway gene products (e.g., an Nmd2p), and to treat genetic disorders involving a nonsense mutation. Assays for testing the effectiveness of compounds such as those described herein are further described below.

Example 12: *In vitro* Screening Assays for Compounds that Bind to Nonsense-mediated Decay Proteins and Genes

In vitro systems can be used to identify compounds that interact with (e.g., bind to) nonsense-mediated

decay pathway proteins or genes encoding those proteins (e.g., a UPF1, UPF3, or NMD2 gene). Such compounds are useful, for example, for modulating the activity of these entities, elaborating their biochemistry, or treating disorders involving nonsense mutations. These compounds can be used in screens for compounds that disrupt normal function, or may themselves disrupt normal function.

Assays to identify compounds that bind nonsense-mediated decay pathway proteins involve preparation of a reaction mixture of the protein and the test compound under conditions sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected.

Screening assays can be performed using a number of methods. For example, a nonsense-mediated RNA decay pathway protein from an organism (e.g., *UPF1* , *NMD2*, *UPF3*, *NMD3*, or *DBP2* protein), peptide, or fusion protein can be immobilized onto a solid phase, reacted with the test compound, and complexes detected by direct or indirect labeling of the test compound. Alternatively, the test compound can be immobilized, reacted with the nonsense-mediated decay pathway molecule, and the complexes detected. Microtiter plates may be used as the solid phase and the immobilized component anchored by covalent or noncovalent interactions. Non-covalent attachment may be achieved by coating the solid phase with a solution containing the molecule and drying. Alternatively, an antibody, for example, one specific for *NMD2* or *UPF1*, is used to anchor the molecule to the solid surface. Such surfaces may be prepared in advance of use, and stored.

In these screening assays, the non-immobilized component is added to the coated surface containing the immobilized component under conditions sufficient to permit interaction between the two components. The

unreacted components are then removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid phase. The detection of the complexes may be accomplished by a number of methods known to those in the art. For example, the nonimmobilized component of the assay may be prelabeled with a radioactive or enzymatic entity and detected using appropriate means. If the non-immobilized entity was not prelabeled, an indirect method is used. For example, if the non-immobilized entity is an Nmd2p, an antibody against the Nmd2p is used to detect the bound molecule, and a secondary, labeled antibody used to detect the entire complex.

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected (e.g., using an immobilized antibody specific for a nonsense-mediated mRNA decay pathway protein).

Cell-based assays can be used to identify compounds that interact with nonsense-mediated mRNA decay pathways proteins. Cell lines that naturally express such proteins or have been genetically engineered to express such proteins (e.g., by transfection or transduction of a UPF1 or NMD2 DNA) can be used. For example, test compounds can be administered to cell cultures and the amount of mRNA derived from a nonsense mutation-containing gene analyzed, e.g., by Northern analysis. An increase in the amount of RNA transcribed from such a gene compared to control cultures that did not contain the test compound indicates that the test compound is an inhibitor of the nonsense-mediated mRNA decay pathway. Similarly, the amount of a polypeptide encoded by a nonsense mutation-containing gene, or the activity of such a polypeptide, can be analyzed in the presence and absence of a test compound. An increase in

the amount or activity of the polypeptide indicates that the test compound is an inhibitor of the nonsense-mediated mRNA decay pathway.

An alternative method of identifying small molecules that inhibit nonsense-mediated mRNA decay involves evaluating the effect of test compounds on yeast cells that contain conditional mutations. The conditional mutations permit growth in the presence (or absence) of a specific factor when the nonsense-mediated mRNA decay pathway is not functional. For example, in the absence of functional NMD2, cells harboring leu2 or tyr7 nonsense mutations gain the ability to grow in the absence of leucine or tyrosine, respectively. Therefore, administration of a test compound that effectively inhibits expression of the wild-type NMD2 gene in a cell harboring one or more of these mutations, permits the cell to grow under the restrictive condition (e.g., in the absence of leucine or tyrosine, or in the presence of canavanine). In such an experiment, for example, yeast cells that contain leu2-3 or tyr7-1 nonsense mutations are grown in the presence and absence of test compounds. Compounds that promote the growth of cells in the presence of leucine or tyrosine, respectively, are candidate compounds to be used as drugs that inhibit the nonsense-mediated mRNA decay pathway. This type of test can also be performed in yeast cells that lack functional NMD2 and express human NMD2 cDNA, thus restoring the nonsense-mediated mRNA decay pathway in a yeast cell with a human gene product to provide an *in vitro* model that can be used to identify candidate compounds that may be effective in humans.

Candidate compounds can also be screened using cells containing a nonsense mutation in the CAN1 gene. Canavanine is a growth inhibitor that requires the presence of arginine permease, a protein encoded by the

CAN1 gene, to enter cells. Cells harboring the can1-100 nonsense mutation are resistant to 100 μg/ml canavanine if they are wild-type for the nonsense-mediated mRNA decay pathway, but sensitive if they harbor an *NMD2* mutation. To screen for candidate compounds that inhibit the nonsense-mediated mRNA decay pathway, yeast cells that have the can1-100 mutation and are wild-type for the nonsense-mediated mRNA decay pathway are incubated in the presence and absence of 100 μg/ml canavanine. If a candidate compound inhibits cell growth in canavanine, the compound is a candidate drug for inhibiting nonsense-mediated mRNA decay.

Candidate inhibitory compounds can be tested in tissue culture cells. For example, nonsense-containing β-globin mRNAs are rapidly degraded in culture cells (Maquat et al., 1981, *Cell* 27:543-553; Maquat, 1995, *RNA* 1:453-465). Such rapid decay would be reversed by candidate drugs that are effective at inhibiting the mRNA decay pathway. Culture cells expressing nonsense mutation-containing globin genes are incubated with a candidate compound. Lysates are prepared from treated and untreated cells and Western blotted according to known methods. The blots are probed with antibodies specific for the amino or carboxy terminus of β-globin and the amount of each quantitated. An increase in the amount of carboxy-terminal β-globin in treated compared to untreated cells indicates that the candidate compound is inhibiting nonsense-mediated mRNA decay and is a candidate for a drug to treat disorders associated with nonsense mutations.

Example 13: Assays for Compounds that Interfere with Upf1p/Nmd2p or Upf3p/Nmd2p Interactions

Assays for compounds that interfere with the interactions of Nmd2p with its binding partners can be based on both biochemical and genetic approaches. In one

biochemical approach, interaction of a Upf1p and an Nmd2p or Upf3p, is monitored using methods described above, or by more automated methods. The latter include the use of devices such as the BIAcore® (Pharmacia Biosensor, Uppsala, Sweden), a surface plasmon resonance detector that measures the interactions of very small amounts of proteins (Szabo et al., (1995) Curr. Opin. Struct. Biol. 5:699-705). The BIAcore provides rapid (e.g., within seconds) graphical output of data indicating whether two molecules have interacted, and the affinity and kinetics of that interaction. Thus, it provides a suitable method of screening for compounds that interfere with the interaction between the molecules of interest. To assay the ability of a candidate compound to interfere with the interaction between, for example, a Nmd2p and a Upf1p, isolated Nmd2p is covalently attached to the suface of a sensor chip. The chip is coated with carboxymethylated dextran and the protein of interest (e.g., an Nmd2p) is linked to the coating via the protein's primary amine groups using carbodiimide coupling. After washing, the chip is inserted into the sensor, and a solution containing the partner protein (in this case, a Upf1p), is pumped over the surface of the chip. Interaction, as surface plasmon resonance, is detected optically in real time (readouts may be collected at 0.1 second intervals). The kinetic rates of association and, after removing unbound free protein (in this case, Upf1p), dissociation are measured. Compounds that are candidates to interfere with the interaction between Nmd2p and Upf1p are added either with Upf1p (to test for interference with association), or after washing out the Upf1p (to test the ability of the candidate compound to enhance the rate of dissociation between Nmd2p and Upf1p). A comparison between the association and dissociation rates of Nmd2p and Upf1p in the presence and absence of the candidate

compound indicates whether the compound affects either rate. Candidate compounds that decrease the rate of association or increase the rate of dissociation are compounds that are to be tested further for their ability to interfere with nonsense-mediated RNA decay. The protocol can also be performed by covalently binding Upf1p to the chip and using Nmd2p as the free partner in the assay. Compounds that interfere with the interaction between Nmd2p and Upf3p can be tested as described for Nmd2p and Upf1p.

A genetic approach using a two-hybrid assay can also be used to screen for compounds that interfere with the interaction between components of the nonsense-mediated mRNA decay pathway. The two-hybrid system is a genetic assay in yeast cells that can detect protein:protein interaction (Fields and Song (1989) Nature 340:245-246; Chien et al., (1991) Proc. Nat. Acad. Sci. USA 88:9578-9582; Fields and Sternglanz, (1994) Trends Genet. 10:286-292). The method is based on the observation that the DNA binding and transcriptional activation functions of the *GAL4* protein (Gal4p) can reside on two distinct chimeric polyeptides and still activate transcription from a *GAL* UAS (upstream activation sequence), provided that the two polypeptides can interact with each other.

To use the two-hybrid assay to screen for candidate compounds that interfere with the interaction betweeen Nmd2p and Upf1p or Upf3p, for example, two plasmids encoding chimeric sequences are constructed. In one plasmid, the nucleic acid sequence containing the entire *UPF1* (or *UPF3*) coding region, or fragments thereof, is fused in-frame to the Gal4p DNA binding domain or the lexA binding domain. The other plasmid contains the *NMD2* gene, or fragments thereof, fused to sequences encoding the Gal4p transcriptional activation

domain (amino acids 768-881). The nonsense-mediated mRNA decay pathway genes can be from a yeast or another organism. Plasmids encoding both hybrid molecules are cotransformed into a *Saccharomyces cerevisiae* strain that contains an integrated *GAL1-lacZ* reporter construct and an integrated *GAL1-HIS3* reporter construct. The transformed yeast are plated and those colonies expressing detectable β-galactosidase activity (blue colonies on X-gal plates) and *HIS3* activity (detected by resistance to 3-aminotriazole [5-80 mM]; 3-AT) are indicative of interaction. The lacZ assay is a quantitative assay for enzymatic activity. The *HIS3* assay provides a colony growth assay, e.g., resistance to different concentrations of aminotriazole. Compounds to be tested for their ability to interfere with the interaction between Nmd2p and Upf1p (or Upf3p) are added to yeast containing the two plasmid constructs in either liquid or solid growth media. Interaction between the two proteins of interest is scored by expression of the lacZ gene or the *HIS3* gene. In the case of the lacZ gene, compounds that specifically interfere with interaction are those that decrease the β-galactosidase activity (i.e., colonies are white, not blue, thus indicating diminished or eliminated activity). The effect of a compound on the interaction can also be detected via HIS3 activity, e.g., compounds that prevent the transformed yeast cells from growing in the presence of 3-AT are candidate compounds for interfering with the interaction between the two proteins (the product of the HIS3 gene antagonizes the latter drug).

Example 14: Assays for Compounds that Ameliorate the Effects of Nonsense-mediated mRNA Decay *in Vivo*

Compounds identified as above, or other candidate compounds that inhibit mRNA-mediated decay pathway

proteins *in vitro* may be useful for treating disorders caused by nonsense mutations. These compounds can be tested in *in vivo* assays, for example, in animal models of genetic disorders involving nonsense mutations. One such model uses mice transgenic for and that express human β-globin genes. These mice have been shown to be subject to nonsense-mediated mRNA decay (Lim et al., Mol. Cell. Biol. 12:1149-1161, 1992).

Candidate compounds predicted to inhibit the nonsense-mediated mRNA decay pathway are administered to animals containing nonsense mutations and assayed for inhibition of the nonsense-mediated mRNA decay pathway. Such assays may be indirect or inferential, for example, inhibition would be indicated by improved health or survival of the animal. Assays may also be direct. For example, inhibition would be indicated by a change in the expression of a disease gene (e.g., nonsense codon-containing gene) as measured, e.g., by Northern analysis of tissue removed from an animal treated with a candidate compound. An increase in the amount of disease gene mRNA present in the sample from treated animals compared to untreated control would indicate that the candidate compound is inhibiting the nonsense-mediated mRNA decay pathway. Similarly, the polypeptide encoded by the disease gene can be measured. For example, an increase in the amount of polypeptide indicates that the candidate compound is inhibiting the nonsense-mediated mRNA decay pathway.

USE

The nonsense-mediated mRNA decay pathway can be inhibited by overexpressing the C-terminal truncated form of an Nmd2p in a cell (such as a yeast cell). Other methods for inhibiting the nonsense-mediated mRNA decay pathway include disruption or mutation of an NMD2 gene or

NMD2 antisense transcript expression. As a result, a transcript for a heterologous protein which contains at least one stop codon within a transcript-destabilizing 5' portion will be specifically stabilized when expressed in a host cell inhibited in a nonsense-mediated mRNA decay pathway. Such stabilization allows translation of the stabilized transcript in a yeast suppressor mutant to produce a full-length peptide with an amino acid inserted at the position of the nonsense codon. The inserted amino acid is specific to the suppressor mutant host in which the heterologous gene and the Nmd2p C-terminus are expressed. The relevant properties of each of the mutant heterologous proteins are compared to the properties of the wild-type protein, and altered heterologous proteins having desired properties are collected. Such properties may include, but are not limited to, protein receptor binding, antibody binding, enzymatic activity, three dimensional structure, and other biological and physical properties known to those of ordinary skill in the arts of biochemistry and protein chemistry.

The invention is also useful in the production of heterologous protein fragments by inserting into the DNA a stop codon within a transcript-destabilizing 5' portion of the coding sequence at a site at which translation is to stop thereby producing an N-terminal protein fragment. This can be done using site-directed mutagenesis. PCR or oligonucleotides containing the desired sequence are used to alter a specfic codon in a gene of interest cloned into an expression plasmid using methods known in the art. Fragments useful in pharmaceutical or other applications can be isolated in large quantities if so desired by techniques well known to those of ordinary skill in the art.

Methods of Treating Disorders Involving Nonsense Mutations

The invention also encompasses the treatment of disorders, especially in mammals, caused by nonsense mutations. A broad range of genetic disorders associated with a nonsense mutation can be treated by the methods described herein. Without limiting the invention by committing to any particular theory, a substantial number of genetic disorders are attributable to the presence of a premature translational termination codon (e.g, nonsense codon) within the coding region of specific genes (e.g., certain cases of β-thalassaemia, breast cancer, polycystic kidney disease I, and Duchenne muscular dystrophy). Table 4 gives examples of specific sites of nonsense mutations associated with cancers such as breast cancer (BRCA1 and BRCA2), colorectal cancer (non-polyposis), retinoblastoma, adrenocortical carcinoma, and Li-Fraumeni syndrome. Table 4 also gives specific examples of nonsense mutations associated with other disorders: Duchenne muscular dystrophy, polycystic kidney disease I, polycystic kidney disease II, Fanconi anemia, haemophilia A, hypercholesterolemia, neurofibromatosis 1, Tay-Sachs disease, glycogen storage disease III, cystic fibrosis, adenomatous polyposis coli, and β-thalassaemia. Many other examples of disorders involving nonsense mutations are known including Cowden disease (Liaw et al., (1997) Nat. Genet. 16:64), Maple syrup urine disease (Fisher et al., (1993) Am. J. Hum. Genet. 52:414), Wilson disease (Thomas et al. (1995) Nature Genet. 9:210), Niemann-Pick disease (Schuchman et al., (1995) Hum. Mut. 6:352), Turcot syndrome (Hamilton et al., (1995) N. Engl. J. Med. 332:839), McArdle disease (Tsujino et al., (1993) N. Engl. J. Med. 329:241), and ornithine transcarbamylase deficiency (Oppliger et al. (1997) Hum. Mutat. 9:409).

TABLE 4

Disease	Codon	Nucleotide	Reference
Breast Cancer (BRCA1)	1	ATGg-ATT	Couch (1996) Hum. Mut. 8:8
	63	TTA-TAA	Inoue (1995) Cancer Res. 55:3521
	484	aGGA-TGA	Couch (1996) Hum. Mut. 8:8
	510	TCA-TGA	Garvin (1996) J. Med. Genet. 57:1284
	526	tCAA-TAA	Friedman (1995) Am. J. Hum. Genet. 57:1284
	563	tCAG-TAG	Shattuck-E (1995) J. Amer. Med. Assoc. 273:535
	639	TTG-TAG	Gayther (1995) Nature Genet. 11:428
	780	tCAG-TAG	Hogervorst (1995) Nature Genet. 10:208
	908	aGAA-TAA	Serova (1996) Am. J. Hum. Genet. 58:42
	1080	TTG-TAG	Gayther (1995) Nature Genet. 11:428

Disease	Codon	Nucleotide	Reference
	1203	cCGA-TGA	Friedman (1994) Nature Genet. 8:399
	1250	cGAG-TAG	Castilla (1994) Nature Genet. 8:387
	1281	tCAG-TAG	Couch (1996) Hum. Mut. 8:8
	1313	cCAG-TAG	Shattuck-E (1995) J. Amer. Med. Assoc. 273:535
	1323	aCAA-TAA	Miki (1994) Science 266:66
	1395	tCAG-TAG	Langston (1996) New Engl. J. Med. 334:137
	1443	gCGA-TGA	Castilla (1994) Nature Genet. 8:387
	1541	aGAG-TAG	Shattuck-E (1995) J. Amer. Med. Assoc. 273:535
	1563	TACc-TAG	Serova (1996) Am. J. Hum. Genet. 58:42
	1725	aGAA-TAA	Merajver (1995) Nature Genet. 9:439
	1727	aAAA-TAA	Gayther (1995) Nature Genet. 11:428

Disease	Codon	Nucleotide	Reference
	1835	cCGA-TGA	Serova (1996) Am. J. Hum. Genet. 58:42
	1837	TGG-TAG	Couch (1996) Hum. Mut. 8:8
Breast Cancer (BRCA2)	194	TGG-TAG	Couch (1996) Nature Genet. 13:123
	1876	gGAA-TAA	Phelan (1996) Nature Genet. 13:120
	1970	TCA-TAA	Gayther (1997) Nature Genet. 15: 103
	2984	TCA-TGA	Gayther (1997) Nature Genet. 15:103
Duchenne Muscular Dystrophy	60	gCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	85	gCAG-TAG	Roberts (1994) Hum. Mut. 4:1
	105	tAAA-TAA	Nigro (1994) Hum. Molec. Genet. 3:1907
	145	cCGA-TGA	Roberts (1994) Hum. Mut. 4:1
	242	aCAA-TAA	Nigro (1992) Hum. Molec. Genet. 1:517

Disease	Codon	Nucleotide	Reference
	250	gGAA-TAA	Roberts (1994) Hum. Mut. 4:1
	354	TGG-TAG	Nigro (1994) Molec. Genet. 3:1907
	480	tGGA-TGA	Roberts (1994) Hum. Mut. 4:1
	491	aCAA-TAA	Kneppers (1995) Hum. Mut. 5:235
	497	tCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	522	tGGA-TGA	Prior (1994) Am. J. Med. Genet. 50:68
	622	TCA-TGA	Nigro (1994) Hum. Molec. Genet. 3:1173
	651	TGG-TAG	Roberts (1994) Hum. Mut. 4:1
	673	tCAG-TAG	Barbieri (1995) Hum. Genet. 96:343
	768	gCGA-TGA	Prior (1993) Hum. Molec. Genet. 2:311
	770	aAAA-TAA	Roberts (1994) Hum. Mut. 4:1
	772	tGAG-TAG	Prior (1993) Hum. Molec. Genet. 2:311
	825	TGGc-TGA	Prior (1995) Am. J. Hum. Genet. 57:22

Disease	Codon	Nucleotide	Reference
	838	tCAG-TAG	Prior (1995) Am. J. Hum. Genet. 57:22
	931	aGAG-TAG	Roberts (1992) Proc. Natl. Acad. Sci. USA 89:2331
	993	aCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	1041	tCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	1063	TGG-TAG	Roberts (1994) Hum. Mut. 4:1
	1087	gCAG-TAG	Nigro (1994) Hum. Molec. Genet. 3:1907
	1102	tCAG-TAG	Barbieri (1996) Eur. J. Hum. Genet. 4:183
	1157	gGAG-TAG	Bulman (1991) Genomics 10:457
	1405	tCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	1459	tCGA-TGA	Prior (1995) Am. J. Hum. Genet. 57:22
	1472	aCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	1642	TTG-TAG	Prior (1995) Am. J. Hum. Genet. 57:22

Disease	Codon	Nucleotide	Reference
	1851	aCAG-TAG	Roberts (1992) Proc. Natl. Acad. Sci. USA 89:2331
	1967	tCGA-TGA	Saad (1993) Hum. Mut. 2:314
	2098	gCGA-TGA	Roberts (1994) Hum. Mut. 4:1
	2125	aCAG-TAG	Roberts (1994) Hum. Mut. 4:1
	2182	aCAG-TAG	Prior (1993) Hum. Mut. 2:192
	2264	cCAG-TAG	Roberts (1994) Hum. Mut. 4:1
	2319	tCAA-TAA	Roberts (1994) Hum. Mut. 4:1
	2757	tGAA-TAA	Prior (1995) Am. J. Hum. Genet. 57:22
	2815	gCAG-TAG	Prior (1995) Am. J. Hum. Genet. 57:22
	2905	aCGA-TGA	Prior (1995) Am. J. Hum. Genet. 57:22
	2972	cCAA-TAA	Tuffery (1996) Eur. J. Hum. Genet. 4:143
	2982	tCGA-TGA	Roberts (1992) Proc. Natl. Acad. Sci. USA 89:2331

Disease	Codon	Nucleotide	Reference
	3024	TGGa-TGA	Prior (1995) Am. J. Hum. Genet. 57:22
	3066	TCG-TAG	Roberts (1994) Hum. Mut. 4:1
	3370	tCGA-TGA	Roberts (1992) Proc. Natl. Acad. Sci. USA 89:2331
	3381	tCGA-TGA	Lenk (1993) Hum. Molec. Genet. 2:1877
	3391	cCGA-TGA	Barbieri (1996) Eur. J. Hum. Genet. 4:183
	3493	cCAG-TAG	Barbieri (1996) Eur. J. Hum. Genet. 4:183
	3635	tCAA-TAA	Prior (1995) Am. J. Hum. Genet. 57:22
Polycystic Kidney Disease I	3818	TACg-TAA	Peral (1996) Hum. Molec. Genet. 5:539
	3837	gCAG-TAG	Peral (1996) Am. J. Hum. Genet. 58:86
	4020	cCGA-TGA	Rossetti (1996) AM. J. Med. Genet. 65:155
	4041	cCAG-TAG	Turco (1995) Hum. Molec. Genet. 4:1331

Disease	Codon	Nucleotide	Reference
	4086	TGTg-TGA	Neophytou (1996) Hum. Genet. 98:437
	4227	cCGA-TGA	Peral (1996) Am. J. Hum. Genet. 58:86
β-Thalassaemia	16	TGG-TAG	Kazazian (1984) EMBO J. 3:593
	16	TGGg-TGA	Aelehla (1990) Hum. Genet. 84:195
	18	cAAG-TAG	Chang (1979) Proc. Natl. Acad. Sci. USA 76:2886
	23	tGAA-TAG	Ghanem (1992) Hum. Mut. 1:229
	27	tGAG-TAG	Baysal (1995) Hemoglobin 19:213
	36	TACc-TAA	Thein (1990) Am. J. Hum. Genet. 47:369
	38	TGG-TAG	Xu (1995) Brit. J. Haematol. 90:960
	38	TGGa-TGA	Boehm (1986) Blood 67:1185
	40	cCAG-TAG	Thecartin (1981) J. Clin. Invest. 68:1012
	44	tGAG-TAG	Atweh (1988) J. Clin. Invest. 82:557

Disease	Codon	Nucleotide	Reference
	62	gAAG-TAG	Gonzales-R (1988) Blood 72:1007
	91	tGAG-TAG	Fucharoen (1990) Brit. J. Haematol. 74:101
	113	TGTg-TGA	Divoky (1993) Brit. J. Haematol. 83:523
	122	aGAA-TAA	Kazazian (1986) Am. J. Hum. Genet. 38:860
	128	gCAG-TAG	Hall (1991) Brit. J. Haematol 79:342
Adenomatous polyposis coli	157	Trp-Term	Olschwang (1993) Am. J. Hum. Genet. 52:273
	805	tCGA-TGA	Doobie (1996) J. Med. Genet. 33:274
	1567	TCA-TGA	Miyoshi (1992) Proc. Natl. Acad. Sci. USA 89:4452
Li-Fraumeni syndrome	213	tCGA-TGA	Frebourg (1995) Am. J. Hum. Genet. 56:608
	306	gCGA-TGA	Cornelis (1997) Hum. Mutat. 9:157
Colorectal cancer, non-polyposis	233	tCAG-TAG	Verlander (1994) Am. J. Hum. Genet. 54:595

Disease	Codon	Nucleotide	Reference
	62	cCAA-TAA	Tannergard (1995) Cancer. Res. 55:6092
	252	TCA-TAA	Papadopoul (1994) Science 263:1625
	714	TGG-TAG	Hutter (1996) J. Med. Genet. 33:636
	252	gCAG-TAG	Liu (1995) Nat. Med. 1:348
	458	TTA-TGA	Liu (1995) Cancer Res. 54:4590
	811	TTA-TGA	Miyaki (1995) J. Mol. Med. 73:515
Fanconi anemia	13	tCAG-TAG	Verlander (1994) Am. J. Hum. Genet. 54:595
	185	aCGA-TGA	Gibson (1993) Hum. Mol. Genet. 2:797
	547	cCGA-TGA	Marer-Orl. (1993) Lancet 342:686
Retinoblastoma	99	TGG-TAG	Blanquet (1995) Hum. Mol. Genet. 4:383
	467	aCGA-TGA	Blanquet (1995) Hum. Mol. Genet. 4:383

Disease	Codon	Nucleotide	Reference
	467	aCGA-TGA	Blanquet (1995) Hum. Mol. Genet. 4:383
Cystic Fibrosis	4	TCG-TAG	Glavac (1993) Hum. Mol. Genet. 2:315
	553	aCGA-TGA	Cutting (1990) Nature 346:366
	1371	tGAA-TAA	Cutting (1992) Am. J. Hum. Genet. 50:1185
Glycogen storage disease III	6	aCAG-TAG	Shen (1996) J. Clin. Invest. 98:352
	680	TGG-TAG	Shen (1996) J. Clin. Invest. 98:352
	1228	tCGA-TGA	Shen (1996) J. Clin. Invest. 98:352
Polycystic kidney disease 2	380	TGG-TAG	Mochizuki (1996) Science 272:1339
	405	aCAA-TAA	Mochizuki (1996) Science 272:1339
	742	tCGA-TGA	Mochizuki (1996) Science 272:1339
Tay-Sachs disease	26	TGGc-TGA	Triggs-Rai (1991) Am. J. Hum. Genet. 49:1041

Disease	Codon	Nucleotide	Reference
	180	TACc-TAG	Drucker (1992) Am. J. Hum. Genet. 51:371
	393	gCGA-TGA	Akli (1991) Genomics 11:124
Neurofibromatosis 1	239	aCAG-TAG	Horn (1996) Electrophoresis 17:1559
	1362	tCGA-TGA	Upadhyaya (1997) Hum. Genet. 99:88
	2518	tGGA-TGA	Heim (1995) Hum. Mol. Genet. 99:674
Hypercholesterol-emia	10	cGAG-TAG	Cenarro (1996) Clin. Genet. 49:180
	210	TGCg-TGA	Gudnason (1993) Arterscl. Thromb. 13:56
	660	TGCc-TGA	Lehrmann (1987) J. Biol. Chem. 262:401
Haemophilia A	-5	gCGA-TGA	Pattinson (1990) Blood 76:2242
	636	TACt-TAG	Becker (1996) Am. J. Hum. Genet. 58:657
	2270	tCAG-TAG	Becker (1996) Am. J. Hum. Genet. 58:657

As described herein, nonsense codons not only interrupt translation, but also promote enhanced decay of transcripts from genes containing nonsense mutations. Based on the yeast paradigm in which inhibition of the nonsense-mediated mRNA decay pathway permits "read-through" and thus increased expression of genes containing nonsense mutations, inhibitors of this pathway, identified as described above are useful for treating disorders involving nonsense mutations.

Therapy is designed to reduce the level of endogenous nonsense-medidated mRNA decay pathway gene expression (e.g., expression of an NMD2, UPF1, UPF3, or homologs thereof) using, e.g., antisense or ribozyme approaches to inhibit or prevent translation of a nonsense-mediated mRNA decay pathway mRNA transcript; triple helix approaches to inhibit transcription of the gene; or targeted homologous recombination to inactivate or "knock out" a gene or its endogenous promoter. The antisense, ribozyme, or DNA constructs described herein can be administered directly to the site containing the target cells; e.g., heart, skeletal muscle, thymus, spleen, and small intestine.

Effective Dose

Toxicity and therapeutic efficacy of the polypeptides of the invention and the compounds that modulate their expression or activity can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Polypeptides or other compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side

effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (that is, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography. Dosages are from about 0.1 to 500 mg per day.

Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets

or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (for example, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (for example, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (for example, magnesium stearate, talc or silica); disintegrants (for example, potato starch or sodium starch glycolate); or wetting agents (for example, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (for example, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (for example, lecithin or acacia); non-aqueous vehicles (for example, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (for example, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, for example,

dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, for example, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The nucleic acids, polypeptides, antibodies, or modulatory compounds of the invention can be administered by any standard route of administration. For example, administration can be parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, opthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, transmucosal, or oral. The modulatory compound can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. Methods for making such formulations are well known and can be found in, for example, "Remington's Pharmaceutical Sciences." It is expected that the preferred route of administration will be intravenous.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the

invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method of determining whether a candidate compound modulates the nonsense-mediated mRNA decay pathway, the method comprising:

a) obtaining a cell containing a mutation in a specific nonsense mutation-containing gene;

b) incubating the cell with the candidate compound under conditions and for a time sufficient for the cell to express nonsense-mediated mRNA decay pathway genes in the absence of the candidate compound; and

c) measuring expression of the nonsense mutation-containing gene, or the activity of a protein expressed by the gene, in the presence and in the absence of the candidate compound, wherein a difference in the expression of the gene or activity of the protein indicates that the compound modulates the nonsense-mediated mRNA decay pathway.

2. The method of claim 1, wherein the cell is a yeast cell containing a nonsense mutation in a gene such that the ability of the cell to grow in a selective medium depends on the functionality of the nonsense-mediated decay pathway.

3. The method of claim 1, wherein the gene containing a nonsense mutation is selected from the group consisting of tyr7, leu2, and CAN1.

4. The method of claim 1, wherein the nonsense-mediated decay pathway gene is NMD2 or a homolog of NMD2.

5. The method of claim 1, wherein the nonsense-mediated decay pathway gene is UPF1 or a homolog of UPF1.

6. The method of claim 1, wherein the nonsense-mediated decay pathway gene is selected from the group consisting of RENT1 and HUPF1.

7. The method of claim 1, wherein the nonsense-mediated decay pathway gene is UPF3 or a homolog of UPF3.

8. The method of claim 1, wherein the cell containing a nonsense mutation is from a mammal.

9. The method of claim 1, wherein the cell containing a nonsense mutation is from a human.

10. The method of claim 1, wherein the candidate compound is a small molecule.

11. The method of claim 1, wherein the candidate molecule is a nucleic acid.

12. A method for treating a mammal having a disorder involving a nonsense mutation, the method comprising administering to the mammal a therapeutically effective amount of a compound that inhibits the nonsense-mediated mRNA decay pathway.

13. The method of claim 12, wherein the mammal is a human.

14. A compound for use in treating a mammal having a disorder involving a nonsense mutation, wherein a therapeutically effective amount of the compound is administered to the mammal to inhibit the nonsense-mediated mRNA decay pathway.

15. The compound of claim 14, wherein the compound causes decreased expression of UPF1 or its homologs.

16. The compound of claim 14, wherein the compound causes decreased activity of Upf1p or its homologs.

17. The compound of claim 14, wherein the compound causes decreased expression of a gene selected from the group consisting of RENT1 or HUPF1.

18. The compound of claim 14, wherein the compound causes decreased expression of NMD2 or its homologs.

19. The compound of claim 14, wherein the compound causes decreased activity of Nmd2p or its homologs.

20. The compound of claim 14, wherein the compound comprises the C-terminal fragment of Nmd2p.

21. The compound of claim 14, wherein the compound causes decreased expression of UPF3.

22. The compound of claim 14, wherein the compound causes decreased activity of Upf3p.

23. The compound of claim 14, wherein the compound is an antisense oligonucleotide.

24. The compound of claim 14, wherein the compound is a ribozyme.

25. The method of claim 12 or compound of claim 14, wherein the disorder is breast cancer, polycystic kidney disease I, polycystic kidney disease II, Niemann-Pick disease, adenomatous polyposis coli, cystic fibrosis, Fanconi's anemia, hemophilia, hypercholesterolemia, neurofibromatosis, ornithine transcarbamylase deficiency, retinoblastoma, glycogen storage disease, McArdle disease, cancer, Tay-Sachs disease, Cowden disease, Wilson disease, or β-thalassaemia.

26. A substantially pure nucleic acid which hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1 or its complementary sequence, wherein said nucleic acid encodes an Nmd2p polypeptide.

27. A substantially pure nucleic acid which hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:3 or its complementary sequence, wherein said nucleic acid encodes a carboxy terminal fragment of an Nmd2p polypeptide that inhibits nonsense-mediated mRNA decay in a cell.

28. A vector comprising the nucleic acid of claim 26 or 27 operably linked to transcriptional regulatory sequences.

```
-361 CCTGAATGACCTTTATCTTAATTATGCACCATCATATAGCGTTTCTATGATCACTACGGGATATTATGATATTGTTAGGGCGTTATATTGAATATTTCTTAGGCCATGAGGATGATATTA

-241 GGGTTATTAATAGGTTTACAATTATATAATTATCACTTGATACGAATTGATGGAGCCTGCTTCTTTTTTTTTTTTTTTCACTTTCTTGGCAGTCACTGAAAAACTGCATT

-121 CGAATACAGGTTTGAGAAACTAATGAGGCCCATATTACTTTACAATGAACAGTAACAATCAACTTAAATGCTTAAATAATCTAATATTGTATCTGCATTGATAATACATTGGACAGAAAT

    -1 TATGGACGTATGTTTGATTTATCTTACTGTGGCCAGATCGGCCTTTCAGTACTTCTAAGGTTTTATACTAACTTCTTTTATTGATCGTTGTAAACTACGGTAACAATTATGTATCAACAG
     1  M  D

   120 GATGGACGGAAAAAAGAATTGCATGATTTGAACACCCGAGCTTGGAATGGCGAAGAAGTCTTTTCCCCTGAAAAGTAAAAAACTGGATTCCAGTATAAAGAGAAACACTGGCTTTTATAAAA
     3  D  G  R  K  K  E  L  H  D  L  N  T  R  A  W  N  G  E  E  V  F  P  L  K  S  K  K  L  D  S  S  I  K  R  N  T  G  F  I  K

   240 AAACTAAAGAAGGGTTTTGTGAAAGGTTCAGAATCTTCATATTGAAAGATTTAAGTGAGGCGTCCTTGGAAAAGTACCTATCAGAGATAATAGTGACGGTAACAGAATGTCTCTGCTAAAT
    43  K  L  K  K  G  F  V  K  G  S  E  S  S  L  L  K  D  L  S  E  A  S  L  E  K  Y  L  S  E  I  I  V  T  V  T  E  C  L  L  N

   360 GTTTTGAATAAAAATGATGACGTAATTGCCGCTGTTGAGATCATAAGTGGACTTCATCAAAGGTTCAATGGCCGATTTACTAGTCCGCTTTTTGGAGCTTTTTTACAAGCTTTTTGAGAAC
    83  V  L  N  K  N  D  D  V  I  A  A  V  E  I  I  S  G  L  H  Q  R  F  N  G  R  F  T  S  P  L  L  G  A  F  L  Q  A  F  E  N

   480 CCCTCTGTTGACATTGAATCCGAAAGAGATGAGCTTCAAAGGATAACCAGAGTTAAAGGTAATCTTCGGTATTTACCGAGCTTTATTTAGTTGGAGTTTTTAGAACATTGGATGATATT
   123  P  S  V  D  I  E  S  E  R  D  E  L  Q  R  I  T  R  V  K  G  N  L  R  V  F  T  E  L  Y  L  V  G  V  F  R  T  L  D  D  I

   600 GAGTCGAAAGATGCTATTCCAAACTTCCTACAGAAGAAACTGGCCGAAAGGATCCGTTGTTATTCAGTATTCTCAGAGAGATTCTTAATTATAAGTTCAAATTGGCTTTACTACCACT
   163  E  S  K  D  A  I  P  N  F  L  Q  K  K  T  G  R  K  D  P  L  L  F  S  I  L  R  E  I  L  N  Y  K  F  K  L  G  F  T  T  T

   720 ATTGCGACCGCATTTATTAGAAATTTGCACCTTTGTTTCGCGACGATGATAATTCTTGGATGATTTAATATATGACTCGAAGTTAAAGGTGCGTTACAGTCTCTGTTTAAGAATTTT
   203  I  A  T  A  F  I  K  K  F  A  P  L  F  R  D  D  D  N  S  W  D  D  L  I  Y  D  S  K  L  K  G  A  L  Q  S  L  F  K  N  F

   840 ATAGACGCCACTTTTGCGAGGGCCACAGAACTGCATAAGAAGGTCAATAAACTGCAAAGAGAACATCAGAAATGCCAAATAAGAACGGGAAAATTGAGAGATGAGTAGTACGTAGAGGAGTAC
   243  I  D  A  T  F  A  R  A  T  E  L  H  K  K  V  N  K  L  Q  R  E  H  Q  K  C  Q  I  R  T  G  K  L  R  D  E  Y  V  E  E  Y
```

FIG. 1A

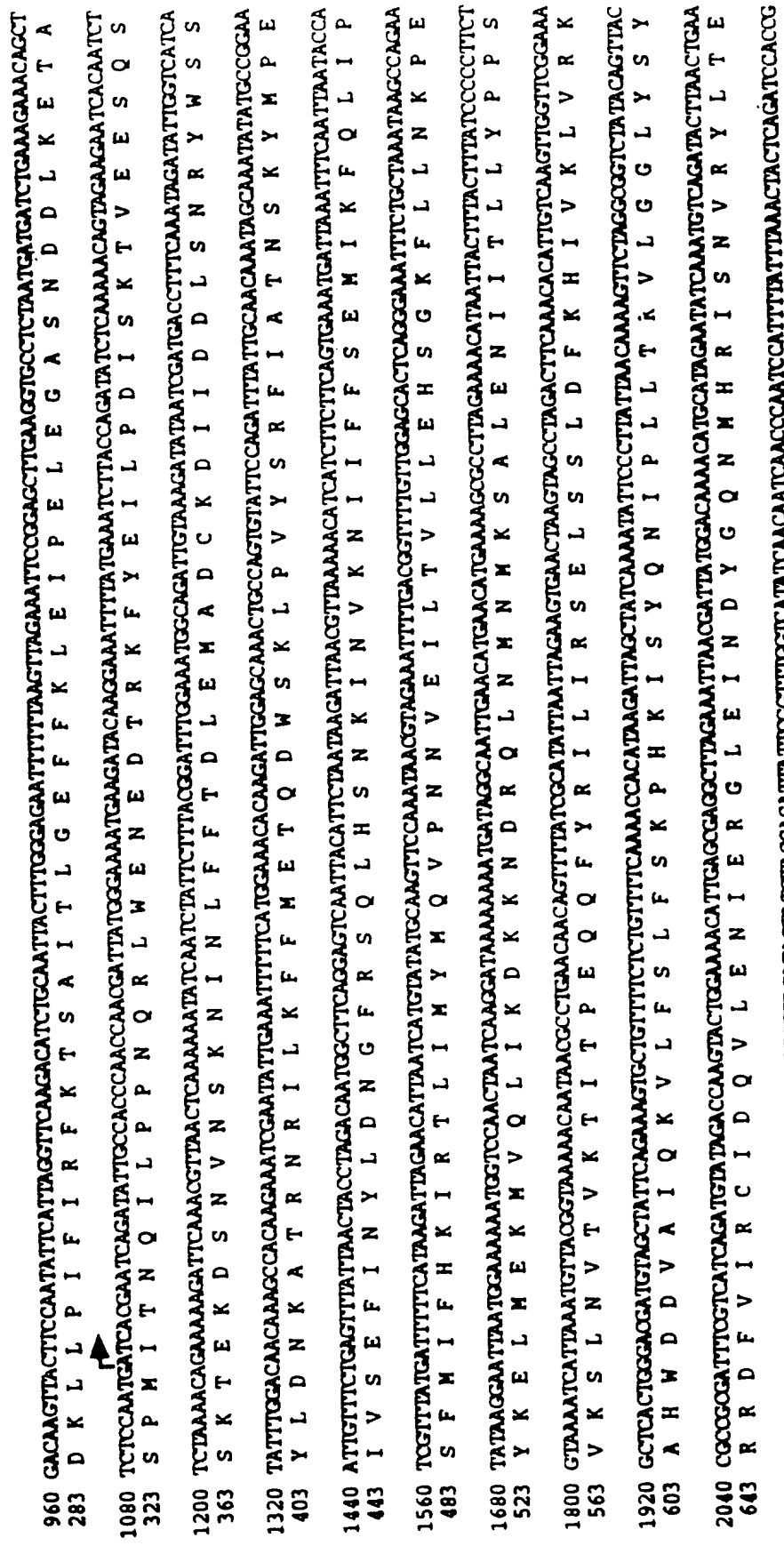
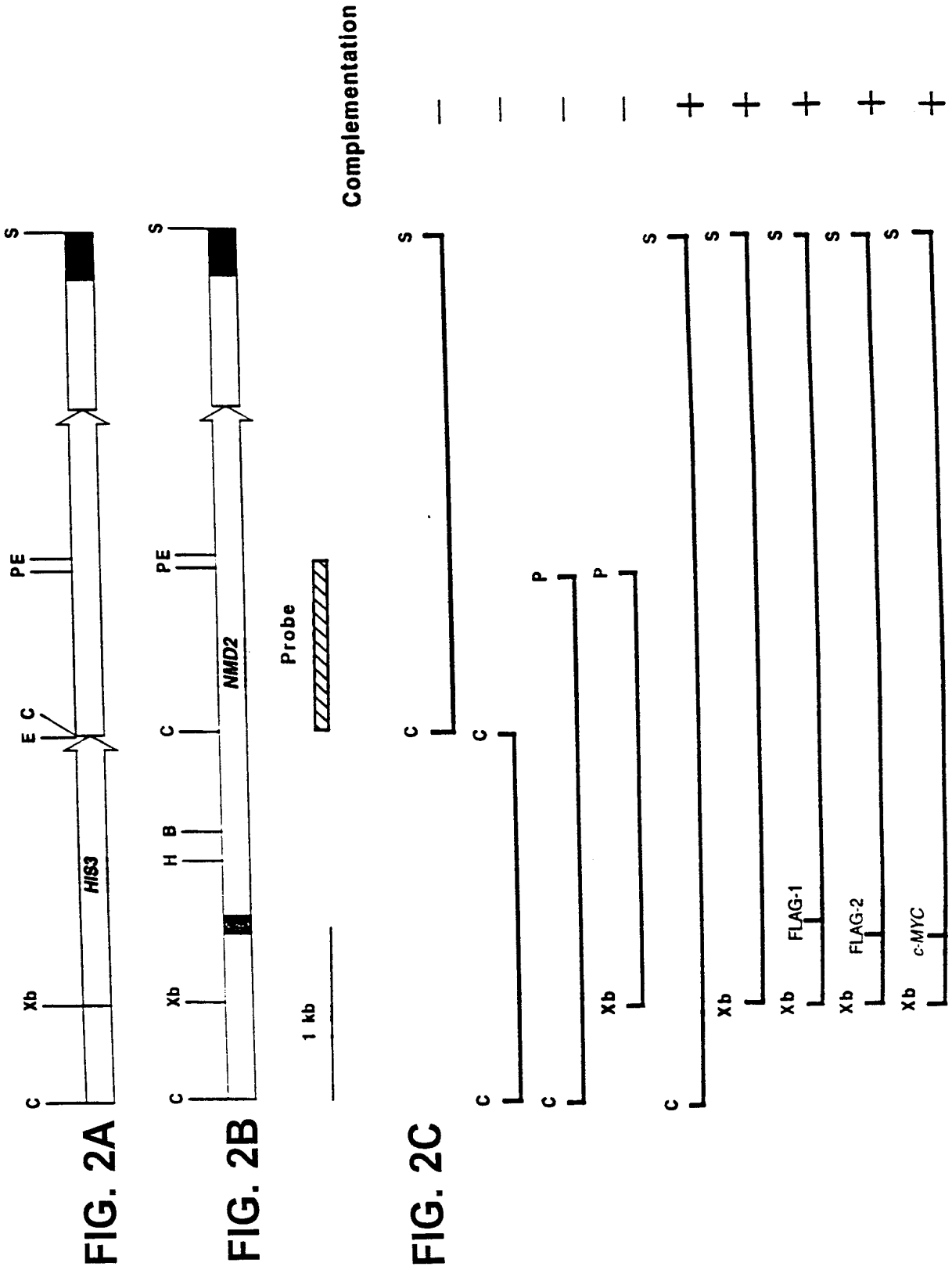

960 GACAAGTTACTTCCAATATTCATTAGGTTCAAGACATCTGCAATTACTTTGGAGAATTTTTTAAGTTAGAAATTCCGGACCTTGAAGGTGCCTCTAATGATCTGAAAGAAACAGCT
283 D K L L P I F I R F K T S A I T L G E F F K L E I P E L E G A S N D D L K E T A
1080 TCTCCAATGATCACGAATCAGATATTGCCACCCAACCAACGATTATGGAAAATGAAGATACAAGGAAATTTTATGAAATCTTACCAGATATCTCAAAACAGTAGAAGAATCACAATCT
323 S P M I T N Q I L P P N Q R L W E N E D T R K F Y E I L P D I S K T V E E S Q S
1200 TCTAAAACAGAAAAAGATTCAAACGTTAACTCAAAAAATATCAATCTATTCTTTACGGATTTGGAAATGGCAGATTGTAAAGATATAATCGATGACCTTTCAAATAGATATTGGTCATCA
363 S K T E K D S N V N S K N I N L F F T D L E M A D C K D I I D D L S N R Y W S S
1320 TATTTGGACAACAAAGCCACACAAGAAATCGAATATTTTTCATGGAACACAAGATTGGAGCAAACTGCCAGTGTATTCCAGATTTATTGCAACAATAGCAAATATATGCCGGAA
403 Y L D N K A T R N R I L K F F M E T Q D W S K L P V Y S R F I A T N S K Y M P E
1440 ATTGTTTCTGAGTTTATTAACTACCTAGACAATGGCTTCAGGAGTCAATTACATTCTAATAAGATTAACGTTAAAAACATCATCTTCTTCAGTGAAATGATTAAATTTCAATTAATACCA
443 I V S E F I N Y L D N G F R S Q L H S N K I N V K N I I F F S E M I K F Q L I P
1560 TCGTTTATGATTTTTCATAAGATTAGAACATTAATCATGTATATGCAAGTTCCAAATAACGTAGAAATTTTGACGGTTTTGTTGGAGCACTCAGGGAAATTTCTGCTAAATAAGCCAGAA
483 S F M I F H K I R T L I M Y M Q V P N N V E I L T V L L E H S G K F L L N K P E
1680 TATAAGGAATTAATGGAAAAATGGTCCAACTAATCAAGGATAAAAAAAATGATAGGCAATTGAACATGAAAGCGCCTTAGAAACATAATTACTTTACTTTATCCCCCTTCT
523 Y K E L M E K M V Q L I K D K K N D R Q L N M M N M K S A L E N I I T L L Y P P S
1800 GTAAATCATTAAATGTTACGGTAAAACAATAACGCCTGAACAACAGTTTTATCGCATATTAATTAGAAGTGAACTAAGTAGCCTAGACTTCAAACACATTGTCAAGTTGGTTCGGAAA
563 V K S L N V T V K T I T P E Q Q F Y R I L I R S E L S S L D F K H I V K L V R K
1920 GCTCACTGGGACGGATGTAGCTATTCAGAAAGTGCTGTTTTCTCTGTTTTCAAAACCACATAAGATTAGCTATCAAAATATTCCCTTATTAACAAAGTTCTAGGCGGTCTATACAGTTAC
603 A H W D D V A I Q K V L F S L F S K P H K I S Y Q N I P L L T K V L G G L Y S Y
2040 CGCCGGAGTTTCGTCATCAGATGTATAGACCAAGTACTGGAAAACATTGAGCGAGGCTTAGAATTAACGATTATGGACAAACATGCATAGAATATCAAATGTCAGATACTTAACTGAA
643 R R D F V I R C I D Q V L E N I E R G L E I N D Y G Q N M H R I S N V R Y L T E
2160 ATATTCAACTTTGAAATGATAAATCCGATGTTTTGTTAGATACTATCTACCACATTATTCGGTTTGGTCATATCAACAATCAACCCAATCCATTTATTTAAACTACTCAGATCCACCG
683 I F N F E M I K S D V L L D T I Y H I I R F G H I N N Q P N P F Y L N Y S D P P

FIG. 1B

```
2280 GATAATTATTTCAGGATTCAACTAGTCACTACAATTCTGTTAAATATCAACAGGACCCCTGCAGCTTTTACTAAGAAATGCAAACTTTTGCTGAGGTTTTTCGAGTATTATACTTTTATT
 723  D  N  Y  F  R  I  Q  L  V  T  T  I  L  L  N  I  N  R  T  P  A  A  F  T  K  K  C  K  L  L  L  R  F  F  E  Y  Y  T  F  I

2400 AAAGAACAACCTTTACCCAAGGAAACAGAATTCAGAGTTTCAAGCACATTTAAAAAATATGAGAATATTTTCGGAAACACTAAATTTGAAAGGTCAGAAAATTTGGTAGAAAGTGCCTCA
 763  K  E  Q  P  L  P  K  E  T  E  F  R  V  S  S  T  F  K  K  Y  E  N  I  F  G  N  T  K  F  E  R  S  E  N  L  V  E  S  A  S

2520 AGGTTGGAAAGTTTACTGAAATCATTAAACGCAATAAAAAGTAAAGACGACAGAGTGAAGGGATCTTCTGCAAGCATTCACAACGGTAAGGAGAGTGCTGTTCCTATCGAGTCAATCACC
 803  R  L  E  S  L  L  K  S  L  N  A  I  K  S  K  D  D  R  V  K  G  S  S  A  S  I  H  N  G  K  E  S  A  V  P  I  E  S  I  T

2640 GAAGATGATGAGGATGAAGATGATGAAAACGACGATGGTGTCGATTTACTAGGAGAAGATGAAGACGCGGAGATAAGTACACCGAACACAGAGTCAGCGCCAGGAAAACATCAGGCAAAG
 843  E  D  D  E  D  E  D  D  E  N  D  D  G  V  D  L  L  G  E  D  E  D  A  E  I  S  T  P  N  T  E  S  A  P  G  K  H  Q  A  K

2760 CAAGACGAAAGTGAAGATGAAGACGATGAGGACGATGACGAGGATGATGACGATGACGATGACGATGATGATGATGATGGAGAAGAAGGCGATGAGGATGATGATGAAGATGATGATGAT
 883  Q  D  E  S  E  D  E  D  D  E  D  D  D  E  D  D  D  D  D  D  D  D  D  D  D  D  G  E  E  G  D  E  D  D  D  E  D  D  D  D

2880 GAGGATGATGATGATGAAGAAGAAGAAGACAGCGACTCTGATTTGGAGTATGGTGGTGATCTTGACGCAGACAGAGATATTGAAATGAAACGAATGTATGAAGAGTACGAGAGAAAACTA
 923  E  D  D  D  D  E  E  E  E  D  S  D  S  D  L  E  Y  G  G  D  L  D  A  D  R  D  I  E  M  K  R  M  Y  E  E  Y  E  R  K  L

3000 AAGGATGAGGAAGAAAGGAAAGCGGAAGAAGAATTGGAAAGGCAATTTCAGAAAATGATGCAAGAATCCATAGACGCAAGGAAAAGCGAAAAGGTTGTTGCCAGTAAAATTCCAGTAATT
 963  K  D  E  E  E  R  K  A  E  E  E  L  E  R  Q  F  Q  K  M  M  Q  E  S  I  D  A  R  K  S  E  K  V  V  A  S  K  I  P  V  I

3120 TCGAAGCCAGTCAGCGTTCAAAAACCTTTATTATTAAAAAAAGTGAAGAACCTTCTTCAAGCAAGGAGACCTACGAAGAGTTATCCAAGCCAAAGAAGATTGCATTTACGTTCTTGACT
1003  S  K  P  V  S  V  Q  K  P  L  L  L  K  K  S  E  E  P  S  S  S  K  E  T  Y  E  E  L  S  K  P  K  K  I  A  F  T  F  L  T

3240 AAAAGCGGTAAGAAGACACAATCAAGAATTTTACAATTACCAACGGATGTGAAATTTGTCTCTGATGTCCTTGAAGAAGAAGAGAAACTAAAAACCGAGCGAAACAAGATTAAAAAGATT
1043  K  S  G  K  K  T  Q  S  R  I  L  Q  L  P  T  D  V  K  F  V  S  D  V  L  E  E  E  E  K  L  K  T  E  R  N  K  I  K  K  I

3360 GTTTTAAAACGTTCTTTCGACTGAGATTCTTTGCGAATATAGTTCTTTAAATTTTTACTATATATGCCCACTTATGTTTGGCTCTATTAAATGGCTACGTGTTTATATAGTACCGTTTAT
1083  V  L  K  R  S  F  D  *        SEQ ID NO: 2(aa 1-1089) SEQ ID NO: 4 (aa 326-1089) SEQ ID NO: 3 (nt 1089-3383)

3480 GACGCTGTATTTTTATTTACACTGCTTTCCAGGAGATTAAAGAGCGGAGTGTTAGTCAACTCTCACGACAACAACAGTTATATCGTCTTCTTTACCACCGCTGTAGTTTTTGCCAGTTAG

3600 CTTAGAAATCTCTTGCGCAAAAACACTGGGGTAATTGGGGTCCTTGCTTAAACTGACAACATTGTCCACAAACTTCTGGGATAATAGCTGTAACTCATCGTTTGTTCTCGCAGCGTTATC
                                                                                                           (SEQ ID NO: 1)
```

FIG. 1C


S
PE
E C
HIS3
Xb
C
FIG. 2A
S
PE
NMD2
C
H B
Xb
C
FIG. 2B
Probe
1 kb
Complementation
FIG. 2C
FLAG-1
FLAG-2
c-MYC

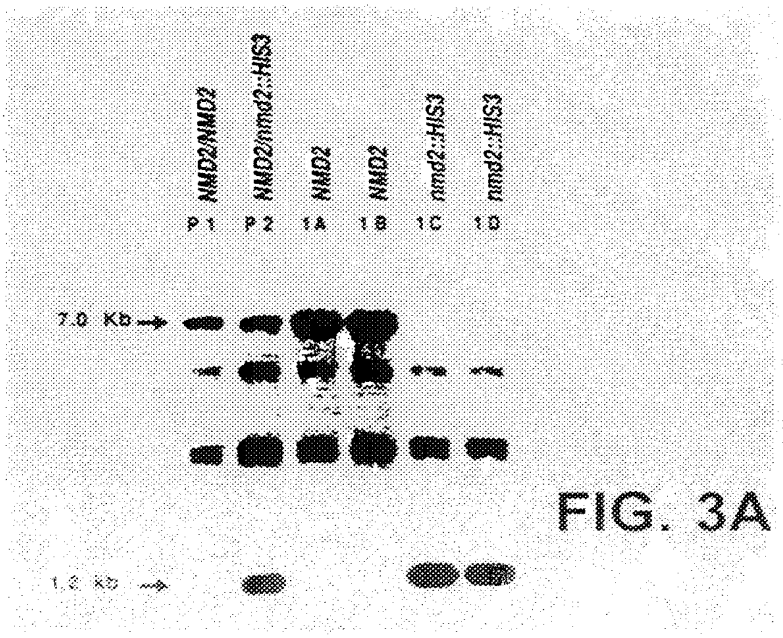


FIG. 3A

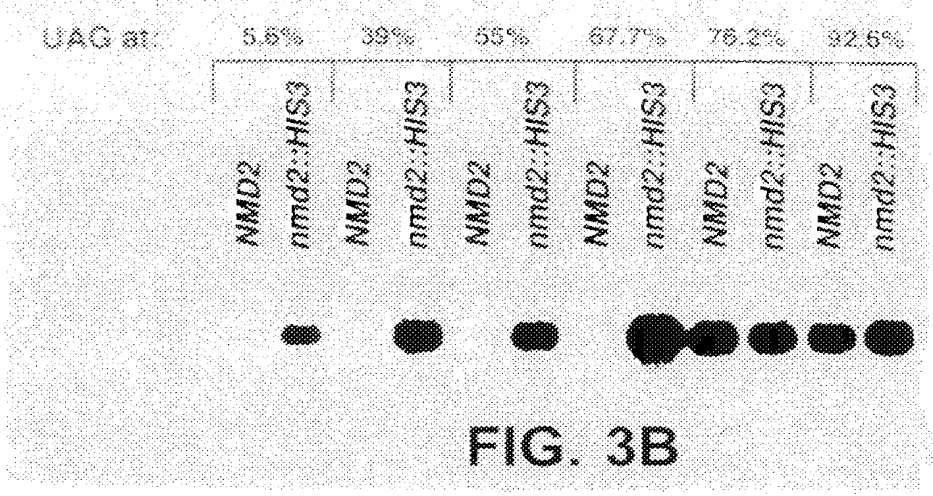


FIG. 3B

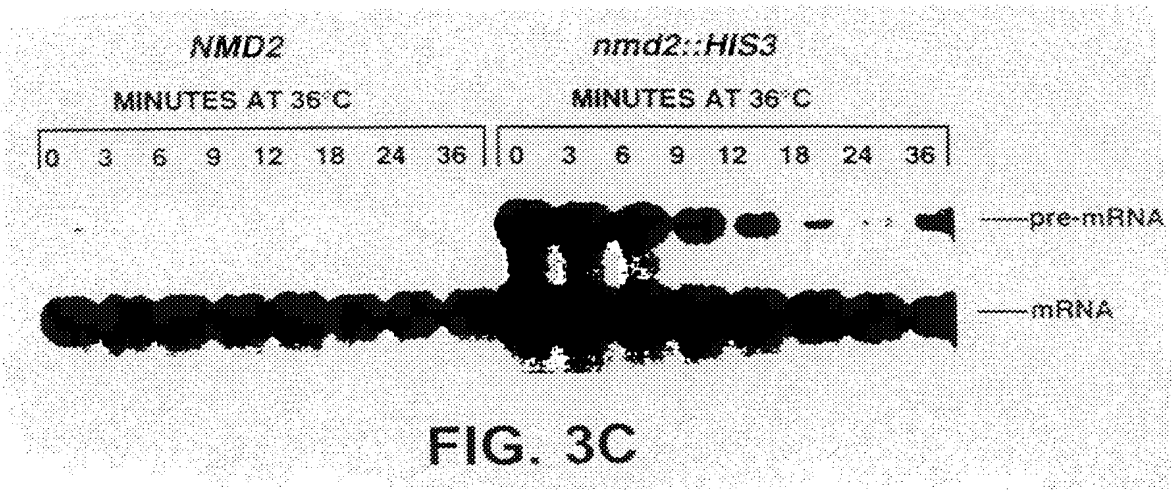


FIG. 3C

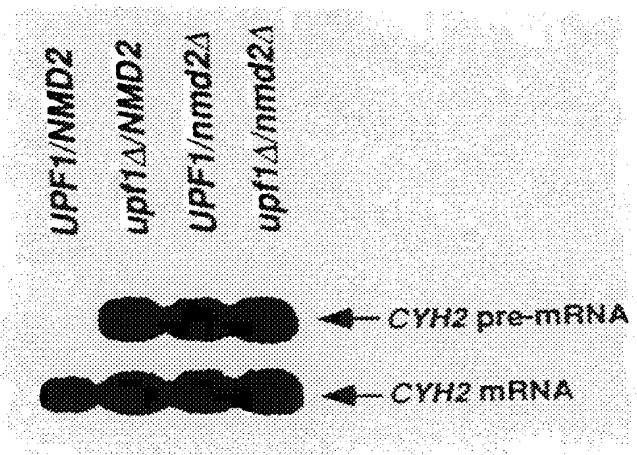

UPF1/NMD2
upf1Δ/NMD2
UPF1/nmd2Δ
upf1Δ/nmd2Δ
CYH2 pre-mRNA
CYH2 mRNA

FIG. 4A

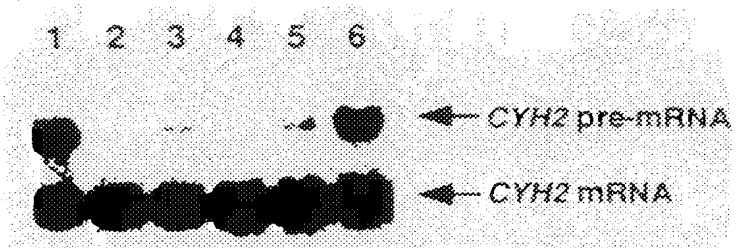

1 2 3 4 5 6
CYH2 pre-mRNA
CYH2 mRNA

FIG. 4B

gaattcatga acgggaaata agaaaaacaa aaagaaaata tacatagtta gttactatcc
actcaataat attaaacgag tgaatgcttt tacttttaac tttagtttaa tcttaattta
cattatttta gtatcatcag tttccctttg cttacttgat ttgggaggga cacctttata
cgctttcgta ctaactgatc aaatgaaaag cttaccagaa acttacgatg ctattgtgaa
ggagaaaaaa aaagcgaaaa gaggcatcgt tttaacgcac actaacagaa gactctattt
ctcttgtcag ccaacaaacg ttgaagattt catcaggaaa gaaggaaggg cagcaagacc
gaatatactt tttatattac atcaatcatt gtcattatca aatggtcggt tccggttctc
acactcctta tgatatatca aactctccat ctgatgtaaa tgtccaaccc gcaacacaac
taaattccac cttggtggag gatgacgatg tagataatca gctatttgaa gaggctcaag
tcactgagac tggattccgt tcgccttcag cttcagacaa ttcatgtgcg tattgtggta
tagattctgc aaagtgtgtc atcaaatgta attcatgtaa gaaatggttt tgtaacacta
aaaacggtac aagcagctcc cacattgtta atcacttagt tttatcccac cataacgtag
tttctttaca tccagattct gacttagggg ataccgtttt ggaatgttat aactgtggac
gtaagaacgt gtttttattg ggatttgttt ccgctaaaag tgaggccgtg gttgttttac
tttgtagaat accttgtgcc cagacgaaaa atgcgaactg ggatactgat caatggcaac
cattaattga agacagacaa cttttatcat gggtcgcaga gcaaccaact gaagaagaaa
aattgaaagc tcgtttaatc actcctagcc aaatttccaa gttggaggca aaatggagat
ccaataaaga cgctacaatt aatgatattg acgccccaga ggaacaggaa gcaatcccac
ctttactatt gagatatcaa gacgcctacg aataccaaag atcttacggg cctttaatca
aattggaggc cgactatgat aaacaactca aggaatctca agctttagaa catatttctg
tttcatggtc cttagcttta aataataggc atttagcatc tttcacttta tctactttcg
aatctaacga gttgaaagtt gccatcggtg atgaaatgat actatggtac tctggcatgc
aacatcctga ttgggaaggt cgtggttaca ttgttcggtt accaaatagc ttccaggaca
cattcacatt agagttaaaa ccaagtaaaa cgccacctcc aacacatttg accactggtt
ttactgctga gttcatctgg aaaggtacct cttatgacag gatgcaagac gcattgaaaa
aatttgccat tgataaaaaa tctatttcag gttatttgta ctataaaatt ttaggccatc
aagtggttga catttcattt gatgtcccat tacctaagga gttttcaatt ccgaattttg
cacaattaaa ctcatcccag tcgaacgctg ttagtcatgt attacaacgt ccgttatctt
taattcaagg cccaccaggc actggtaaaa cagttacttc agcaacgatt gtgtatcacc
tttccaaaat acacaaggat agaatattgg tgtgtgcccc atcaaacgtt gctgtagatc
atttggctgc caaattacgt gacttgggtt taaaagttgt tagacttacc gcgaaaagta
gagaagatgt ggagagttcc gtctccaact tagcattgca taatttggtt ggccgtggtg
ctaaagggga attaaaaaac ctattaaagt taaaggatga agttggcgaa ttatctgctt
ctgatacaaa acggtttgtt aaattagtaa ggaaaacaga agcagaaatt ctcaataagg
cagatgtcgt atgttgcaca tgtgttggtg ctggtgataa gcgcttagac actaaattta
ggactgtgtt aattgatgaa agtactcaag cttctgagcc ggaatgttta atcccaatcg
ttaaaggtgc gaaacaagtt atacttgttg gtgatcacca gcaactgggc ccagtcatat
tggaacgaaa ggcggcagac gctggtttga aacaatctct ctttgaaaga ttaatctctc
taggccacgt accgattcgt ttggaagttc aataccgtat gaatccttat ttgagtgagt
ttccaagtaa catgttttat gaaggcagcc tacaaaatgg tgtaacgatt gaacagcgta
ccgttcccaa cagcaaattc ccatggccaa ttcgcggtat accaatgatg ttttgggcca
attacggtag agaggagatt tctgctaacg gtacttcctt cttaaacaga attgaagcca
tgaattgtga acgaatcatc actaaacttt tcagagacgg tgtcaagccc gagcaaattg
gtgttatcac accatatgag ggacaaagag cttatatttt acaatatatg caaatgaatg
gttcattgga taaggatttg tatatcaaag tggaagttgc ctcagttgat gcattccaag
gtcgtgaaaa ggattacata atcttatcgt gtgttcgtgc caatgaacaa caggccattg
gtttcttacg tgatcctcgt cgtctaaacg tgggtctaac ccgtgccaaa tatggtctag
ttattcttgg taatcctaga tctttggcaa gaaacacatt atggaaccat ctgttaatcc
acttcagaga gaagggttgt ttagtcgaag gtacgttgga taacttacag ttatgcactg
ttcaattagt tcgtcctcag ccaagaaaga ctgaacggcc aatgaacgct caatttaacg
tagaatctga aatgggtgac tttccgaagt tccaggattt tgatgcacag agtatggtgt
cattcagtgg tcaaattggg gactttggta atgcatttgt tgacaacaca gaactttctt

FIG. 5A

cttacatcaa taatgaatat tggaattttg agaattttaa aagtgctttt tctcaaaagc
aaaatcgcaa tgaaattgac gatagaaatt tgtaccagga ggaggcttct catttgaact
ctaacttcgc gagagagtta cagagagaag aacaaaagca tgaattgtca aaagacttca
gcaatttggg aatataattc ggtgaaccct gttaaaataa aatgttaaac ttggcttgtg
atacaaaacg gctcaaccgt gaaatgagcg ctgcaaaatt attcgagata gactcgcaat
ttgcacaatt gtaacctgaa aaatttttt actttccgg aggtgcatct atcattacag
tatgtgataa aggggcatgg acttgatatc ctagcctact aatctctttg ctaaaacatg
ttgcaa (SEQ ID NO:7)

FIG. 5B

MVGSGSHTPYDISNSPSDVNVQPATQLNSTLVEDDDVDNQLFEE
AQVTETGFRSPSASDNSCAYCGIDSAKCVIKCNSCKKWFCNTKNGTSSSHIVNHLVLS
HHNVVSLHPDSDLGDTVLECYNCGRKNVFLLGFVSAKSEAVVVLLCRIPCAQTKNANW
DTDQWQPLIEDRQLLSWVAEQPTEEEKLKARLITPSQISKLEAKWRSNKDATINDIDA
PEEQEAIPPLLLRYQDAYEYQRSYGPLIKLEADYDKQLKESQALEHISVSWSLALNNR
HLASFTLSTFESNELKVAIGDEMILWYSGMQHPDWEGRGYIVRLPNSFQDTFTLELKP
SKTPPPTHLTTGFTAEFIWKGTSYDRMQDALKKFAIDKKSISGYLYYKILGHQVVDIS
FDVPLPKEFSIPNFAQLNSSQSNAVSHVLQRPLSLIQGPPGTGKTVTSATIVYHLSKI
HKDRILVCAPSNVAVDHLAAKLRDLGLKVVRLTAKSREDVESSVSNLALHNLVGRGAK
GELKNLLKLKDEVGELSASDTKRFVKLVRKTEAEILNKADVVCCTCVGAGDKRLDTKF
RTVLIDESTQASEPECLIPIVKGAKQVILVGDHQQLGPVILERKAADAGLKQSLFERL
ISLGHVPIRLEVQYRMNPYLSEFPSNMFYEGSLQNGVTIEQRTVPNSKFPWPIRGIPM
MFWANYGREEISANGTSFLNRIEAMNCERIITKLFRDGVKPEQIGVITPYEGQRAYIL
QYMQMNGSLDKDLYIKVEVASVDAFQGREKDYIILSCVRANEQQAIGFLRDPRRLNVG
LTRAKYGLVILGNPRSLARNTLWNHLLIHFREKGCLVEGTLDNLQLCTVQLVRPQPRK
TERPMNAQFNVESEMGDFPKFQDFDAQSMVSFSGQIGDFGNAFVDNTELSSYINNEYW
NFENFKSAFSQKQNRNEIDDRNLYQEEASHLNSNFARELQREEQKHELSKDFSNLGI
(SEQ ID NO:8)

FIG. 6

gaattctact tgataggatt ttattgccgt ctttttctat aagttctata tcctcagtat
cgtcttcttc ctcgtcttcc tcctcatgac tagtagagtg agaagatgaa tgatcagaat
tattgatgat ttcgttatct tctaaagtca ccaaatcccc atgtaaatca tccaatgcag
tgttcatatt aatgattgag tagattggta catatgctat ttcggaagac ttttgttatt
ctatggttta tcatcccttt atttatttttg tgtattgttt gctgatcaaa agttgaaaat
ttttcgccta aaaagtaaga tacaaaagaa aatattgtcg atgattattg catgaatata
tcagcaaaga ggaaaggaaa acctactgag ggacttacat ttctgctgaa atatatagta
atctatcatg agcaatgtgg ctggggaatt gaagaatagt gaggggaaaa agaaaggcag
gggaaatagg tatcataaca agaacagagg aaaaagtaag aatgagacgg tagatcctaa
aaagaatgag aataaggtta ataatgctac taatgctacc cacaacaata gcaaaggcag
aaggaataac aagaaaagga acagagagta ttataactat aaaagaaagg ctagattggg
taaatcaacc gagaatgaag gatttaagct tgttattaga ttgctacctc caaatttgac
tgcagatgaa ttttttgcca tcttacgaga taataataac gatgatggtg ataagcaaga
tatccagggt aaactcaagt acagtgactg gtgttttttt gaaggtcatt attctagtaa
agtattcaaa aactcgacat attctcggtg caatttcttg ttcgacaact tatcagactt
ggaaaaatgc gcaaatttca ttaaaacttg taaattcatt gataataagg ataatattac
aattccagat atgaaactgt cgccctacgt aaagaaattc actcaaacat caaaaaagga
tgccgcgcta gtaggaacaa ttgaagaaga cgaaattttt aaaacattta tgaattcaat
gaaacagctg aatgaaaatg acgagtactc attccaagat tttagcgtat tgaaatcttt
agaaaaagaa ttctcaaaaa gcatagagtt agaaaataaa atagcagaaa gaacagaaag
ggtgttaaca gagctggttg gaactggtga taaggtcaag aataagaaca aaaagaagaa
aaataaaaac gccaaaaaga aattcaaaga agaggaagca tccgctaaga taccaaagaa
aaaacggaac agaggcaaga agaagcgtga aaatcgtgaa aaaagcacca tttctaagac
caagaacagt aatgtggtta ttattgagga agcgggtaaa gaggttttga aacaaaggaa
gaagaaaatg cttttgcaag agaagttaaa aatatcaaac tcctctcagc ctcagtcatc
atccgctcaa acccagccgt cgttccaacc taaagaaaac cttttcgtac cacgggtaaa
aattttgcat cgtgatgata ccaagaagta gtaaaagctc atggcttctt atatattata
tatggaatac atttataata aaataataag aattatatat tttatgatta tattattaca
taaagtattc cccattataa attctgagtt tcgtatttaa tgatttttca atgaatattt
aaaataataa aatatatgaa atgttcatat acaatgaaat tgtcatgaag aaagatgact
ccaagtatcg tttataaatc gtcgagaaaa agattatgaa gttggttaac tttttaaaaa
acgtgcgcaa tgagcaggtt accatagaac taaaaaacgg taccaccgtt tggggtacac
tgcagtcggt atcaccacaa atgaatgcta tcttaactga (SEQ ID NO:9)

FIG. 7

MSNVAGELKNSEGKKKGRGNRYHNKNRGKSKNETVDPKKNENKV
NNATNATHNNSKGRRNNKKRNREYYNYKRKARLGKSTENEGFKLVIRLLPPNLTADEF
FAILRDNNNDDGDKQDIQGKLKYSDWCFFEGHYSSKVFKNSTYSRCNFLFDNLSDLEK
CANFIKTCKFIDNKDNITIPDMKLSPYVKKFTQTSKKDAALVGTIEEDEIFKTFMNSM
KQLNENDEYSFQDFSVLKSLEKEFSKSIELENKIAERTERVLTELVGTGDKVKNKNKK
KKNKNAKKKFKEEEASAKIPKKKRNRGKKKRENREKSTISKTKNSNVVIIEEAGKEVL
KQRKKKMLLQEKLKISNSSQPQSSSAQTQPSFQPKENLFVPRVKILHRDDTKK
(SEQ ID NO:10)

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/22365

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12N 5/06, 5/08, 1/16; C07H 21/00

US CL :435/6, 255.1, 325; 536/24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 255.1, 325; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis, WPIDS
search terms: nonsense mediated RNA decay, inhibitor, drug, screen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DARLING et al. Premature Termination Codons Are Present on Both Alleles of the Bullous Pemphigoid Antigen 2/Type XVII Collagen Gene in Five Austrian Families with Generalized Atrophic Benign Epidermolysis Bullosa. J. Invest. Dermatol. April 1997, Vol. 108, Number 4, pages 463-468, especially pages 466-467.	1, 4-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 JANUARY 1999	Date of mailing of the international search report 26 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN S. BRUSCA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/22365

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/22365

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group 1, claim(s) 1-11, drawn to a method of assay of modulators of nonsense-mediated mRNA decay.
Group 2, claim(s) 12, 13, and 25, drawn to a method of therapeutic use of a compound that inhibits nonsense-mediated mRNA decay.
Group 3, claim(s) 14-24, drawn to a therapeutic compound that modulates nonsense-mediated mRNA decay.
Group 4, claims 26-28, drawn to a gene encoding nmd2P protein and vectors comprising the gene.

The inventions listed as Groups 1-4 do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The assay method of Group 1, the first appearing method, is not related to the therapeutic method of Group 2 or the modulator of Group 3 or the nmd2P gene of Group 4 because the method of group I is not a therapeutic method and does not employ a therapeutic compound. Group 2 is the second appearing method, and is related to the therapeutic product of Group 3 as a process of using the product, and therefore does not have unity with the first appearing method. The therapeutic method of Group 2 is not related to the nmd2P gene of Group 4 because the nmd2P gene encodes a protein that facilitates nonsense-mediated mRNA decay, and the therapeutic method requires use of a compound that modulates nonsense-mediated decay with beneficial results. The therapeutic product of Group 3 is not related to the nmd2P product of Group 4 because the nmd2P gene is not a therapeutic product.